

**BIOCHEMICAL AND MOLECULAR CHARACTERIZATION OF
A- AND B-TYPE STARCH GRANULES IN WHEAT (*Triticum
aestivum* L.) ENDOSPERM**

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By

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ABSTRACT

Mature wheat endosperm contain A- (diameter $> 10\ \mu\text{m}$) and B-type (diameter $< 10\ \mu\text{m}$) starch granules, and show a bimodal granule size-distribution. Because the two starch granule types differ significantly in chemical composition and functional properties, wheat cultivars with a unimodal size-distribution of either A- or B-type starch granules would be very useful for the food and non-food industries. Breeding such wheat cultivars may be accelerated by the molecular and biochemical characterization of A- and B-type starch granules. Thus, the objectives of this study were the development of a method to purify A- and B-type starch granules, and the analysis of proteins and genes that may be related to the biosynthesis of the two starch granule types.

Two methods, microsieving and centrifugal sedimentation through aqueous solutions of sucrose, maltose or Percoll were used to separate A- and B-type starch granules. Homogeneous A- and B-type starch granule populations, representing their counterparts in mature wheat endosperm, were obtained by centrifugation through two Percoll solutions (70% and 100%). Two starch granule-bound proteins, SGP-140 and SGP-145 were found to be preferentially associated with A-type starch granules in developing and mature wheat endosperm. Both SGP-140 and SGP-145 were different variants of SBEIc, a 152 kD isoform of wheat starch branching enzyme, and localized to the endosperm starch granules. The preferential association of SGP-140 and SGP-145 with A-type starch granules was correlated to the growth of wheat A-type granules, and related to the occurrence of the bimodal starch granule size-distribution in barley, rye and triticale endosperm.

Starch synthase I has been implicated in the size determination of A-type starch granules in barley. However, it is not known whether wheat starch synthase I (wSSI) has a similar function. Thus, biochemical and molecular characterization of wheat A- and B-type starch granules makes it necessary to clone and characterize wSSI. In developing wheat endosperm, wSSI was present at similar levels in starch granules throughout development, while in soluble fractions, wSSI was not detected at five days-post-anthesis (DPA), and had highest concentrations from 10 to 15 DPA. Analysis on a single wheat kernel basis showed that wSSI had similar concentrations in soluble fractions from 15 to 25 DPA, and much more wSSI was distributed in starch granules than in soluble endosperm fractions. Furthermore, wSSI accounted for approximately 65% of the total soluble starch synthase activity in wheat endosperm. Two full-length cDNA clones encoding wSSI were isolated from a wheat cDNA library. Expressed in *E.coli* strain RH98 with glycogen synthase deficiency, the wSSI cDNA produced an active starch synthase, and complemented the glycogen synthase deficiency. *wSsI* transcript was detected in leaf, stem, root, pre-anthesis floret, ovary and pollen tissues. In endosperm, the expression of *wSsI* was higher at 5 - 10 DPA than at 15 - 25 DPA.

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LIST OF ABBREVIATIONS

ADP	Adenosine diphosphate
AGPase	ADPglucose pyrophosphorylase
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
cDNA	Complementary DNA
cpm	Counts per minute
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytosine triphosphate
dGTP	Deoxyguanine triphosphate
dTTP	Deoxythymidine triphosphate
DBE	Debranching enzyme
dp	Degree of polymerization
2D-PAGE	Two-dimensional polyacrylamide gel electrophoresis
DPA	Days-post-anthesis
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
GBSS	Granule bound starch synthase
IPTG	Isopropyl- β -D-thiogalactopyranoside
kb	Kilo base
kDa	Kilo Dalton
LB	Luria-Broth
mRNA	Messenger RNA
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
pfu	Plaque forming units
PVP	Polyvinylpyrrolidone
RNA	Ribonucleic acid
SBE	Starch branching enzyme
SDS	Sodium dodecyl sulphate
SGP	Starch granule-bound protein
SS	Starch synthase
Tris	Tris (hydroxymethyl) amino methane
UDP	Uridine diphosphate
UDPG	UDPglucose: protein transglucosylase
X-gal	5-bromo-4-chloro-3-indolyl- β -D-galactoside

CHAPTER 1.

INTRODUCTION

Wheat (*Triticum aestivum* L.) is one of the most important staple foods in the world. Starch is the predominant component of wheat endosperm (Hucl and Chibbar, 1996), and constitutes the major source of carbohydrate in the human diet. Starch is present as discrete, water-insoluble granules in plastids. Two types of starch granules are found in mature wheat endosperm. The A-type starch granules are 10-35 μm in diameter and lenticular in shape; while the B-type starch granules are less than 10 μm in diameter and spherical or polygonal in shape (Evers, 1973). A-type starch granules account for only 3% of the total starch granule number in a wheat endosperm, but contribute more than 70% of the total endosperm starch weight.

Wheat A- and B-type starch granules are distinct in their development, chemical composition and functional properties. First, A-type starch granules are synthesized in amyloplast (A-type amyloplast) at four to five days-post-anthesis (DPA), while B-type granules are initiated within the stroma of A-type amyloplast at 12-14 DPA, and released into the cytoplasm late during grain development (Parker, 1985). Second, B-type starch granules usually contain less amylose, but a higher amount of lipid and granule-associated protein than A-type granules. Third, both types of starch granules differ significantly in gelatinization properties, pasting characteristics, and baking applications (Seib, 1994). Genetic analysis suggests that the proportion of B-type starch granules in wheat endosperm is determined by additive genes with a narrow-sense heritability of

0.59 (Stoddard, 1998). Quantitative trait loci (QTL) affecting the overall size and the proportion of A-type starch granules are mapped on chromosome 2 in barley (*Hordeum vulgare* L.) (Borém et al., 1999). However, there are no reports concerning the biochemical and molecular characterization of A- and B-type starch granules in wheat.

The differing chemical composition and functional properties of A- and B-type starch granules result in the two types of starch granules being utilized differently in both food and non-food industries. A-type starch granules are preferred for bread making, and are desirable in any rapid industrial process, such as the manufacture of starch and gluten from flour. because B-type starch granules are usually washed away, thus resulting in low yield and increased manufacturing costs (Bechtel et al., 1990; Stoddard, 1999). Starch with predominantly B-type granules can be used as fat substitutes (Lim et al., 1992), while starch with a high percentage of A-type starch granules has applications in the manufacture of biodegradable plastic film and carbonless copy paper (Nachtergaele and Van Nuffel, 1989). Therefore, wheat cultivars with either A- or B-type starch granules would be very useful both in food and non-food industries. In order to develop such wheat cultivars, the molecular and biochemical characterization of wheat A- and B-type starch granules is essential.

The objective of this study was to investigate the molecular and biochemical mechanisms that control the biosynthesis of A- and B-type starch granules during wheat endosperm development. Starch synthases (SS), starch branching enzymes (SBE) and starch debranching enzymes (DBE) participate in the biogenesis of plant starch granules (Ball et al. 1996; Preiss and Sivak, 1998). Each of these starch biosynthetic enzymes exists in multiple isoforms, and mutations inactivating any of these enzymes result in the

modification of starch structure and/or granule morphology (Bhattacharyya et al., 1990; Mouille et al., 1996; Craig et al., 1998; Yamamori, 1998; Edwards et al., 1999). Thus, it is conceivable that different isoforms of SS, SBE, and DBE may play important roles in the biosynthesis of A- and B-type starch granules in developing wheat endosperm. In the barley shrunken (*shx*) mutant, the overall sizes of A-type starch granules are reduced, and the starch granule size-distribution is apparently unimodal rather than bimodal as in the wild type. Although several enzymes of the starch synthetic pathway were affected, starch synthase I was suggested as the primary mutation site in the *shx* mutant (Schulman and Ahokas, 1990; Tyynelä and Schulman, 1993; Tyynelä et al., 1995). Therefore, one approach utilized in this study was to compare the isoforms of starch synthetic enzymes during the development of A- and B-type starch granules, and to determine if there are some isoforms that are related to the biosynthesis of A- or B-type starch granules.

No mutant with altered starch granule size distribution, analogous to the *shx* endosperm in barley, has been reported in wheat. Thus, it is difficult to determine which of the multiple isoforms of starch synthetic enzymes are related to the biosynthesis of A- and B-type starch granules. The only available starting materials are A- and B-type starch granules themselves. It is well documented that starch granule-bound proteins (SGP) play important roles in starch granule synthesis. The 60 kD SGP has been shown to be a starch granule-bound starch synthase (GBSS I), and responsible for the synthesis of amylose in starch granules (Shure et al., 1983). In a wheat line lacking SGP1, a starch granule-bound starch synthase II (SS II), large starch granules were mostly deformed, and a novel starch with increased iodine binding capacity was produced (Yamamori,

1998). Therefore, it was considered prudent to investigate whether some SGP are related to the biosynthesis of A- and B-type starch granules.

Purified A- and B-type starch granules are essential for precise analysis of SGP from the two types of starch granules. Several procedures, including microsieving (Bathgate and Palmer, 1972), sedimentation in water (Bond et al., 1979; Meredith, 1981), and elutriation (MacGregor, 1979) have been developed to separate A- and B-type granules. However, none of the three methods can give a complete separation of A- and B-type starch granules (Eliasson and Karlsson, 1983). Thus, there is a need for a method to effectively purify A- and B-type starch granules.

In summary, the objectives of this study were to: 1) develop a method to purify A- and B-type starch granules; 2) analyze proteins and genes that may be related to the biosynthesis of A- and B-type starch granules. Several experiments have been successfully completed and written into three papers. 1) Separation and characterization of A- and B-type starch granules in wheat endosperm. 2) Starch branching enzymes preferentially associated with A-type starch granules in wheat endosperm; 3) Starch synthase I is the major soluble starch synthase in wheat endosperm: molecular cloning and characterization of wheat starch synthase I.

CHAPTER 2.

LITERATURE REVIEW

2.1 Plant Starch Granules: Structure and Biosynthesis

2.1.1 Plant starch granules

Starch (amylum), a glucose polymer, is the predominant food reserve in plants, and constitutes the main source of carbohydrate in the human diet. It is composed of two glucan polymers: amylose and amylopectin. Amylose makes up to 20 - 30% of starch, and consists of predominantly linear chains of about 10^3 - 10^4 glucose residues linked by $\alpha(1, 4)$ glucosidic bonds. The linear chains in amylose are usually branched at a low level by $\alpha(1, 6)$ linkages (about one branch per 1000 residues) (Hizukuri et al, 1981; Shannon and Garwood, 1984). Amylopectin constitutes 70 – 80% of starch, and is comprised of short $\alpha(1, 4)$ glucan chains with an average length of 17 – 23 glucose residues, which are joined by frequent $\alpha(1, 6)$ linkages. Thus, amylopectin is heavily branched and has a very high molecular weight, about 10^7 - 10^9 (Hizukuri et al., 1983).

Amylopectin and amylose are usually packed as discrete, water-insoluble starch granules in plastids. Starch granules are classified as transitory or reserve granules (Badenhuizen, 1969). Transitory starch granules accumulate in chloroplasts in leaves for only a short period of time before their degradation, and lack species-specific shape. Reserve starch granules are usually present in amyloplasts in storage organs, such as seeds, tubers and roots (Badenhuizen, 1965). An amyloplast may contain one or several starch granules depending on the plant species. In wheat, maize (*Zea mays* L.), rye

(*Secale cereals* L.), barley (*Hordeum vulgare* L.), sorghum (*Sorghum bicolor* L.), potato (*Solanum tuberosum* L.), and pea (*Pisum sativum* L.), an amyloplast contains one starch granule, called a simple granule. In contrast, in rice (*Oryza sativa* L.), cassava (*Manihot utilissima* L.) and sweet potato (*Ipomoea batatas* L.), each amyloplast contains two or more starch granules, which are referred to as compound starch granules (Shannon and Garwood, 1984). Compared with transitory starch granules, those produced in storage organs are much more commercially valuable, and have species-specific shape and size. Thus, this review will focus on reserve starch granules.

Reserve starch granules vary widely in granule sizes and size-distribution with plant species. Canna (*Canna edulis* L.) and white potato have the largest starch granules (120 μm), while those from rice and buckwheat (*Fagopyrum esculentum* L.) are the smallest (2 μm) (Kerr, 1951; Shannon and Garwood, 1984). Further, starch granules from the same plant species can vary in diameter from 0.25 μm to over 100 μm (Brautlecht, 1953). In many plant species, such as maize, rice, oat (*Avena sativa* L.) and potato, the starch granule populations show a continuous range of granule sizes, and thus have a unimodal starch granule size-distribution. In wheat, barley, rye, and triticale (*X Triticosecale* Wittmack) endosperm, two starch granule populations with distinct sizes and shapes are present: the large lenticular shaped granules and the small spherical granules. Therefore, these plant species have a bimodal starch granule size-distribution (Shannon and Garwood, 1984).

2.1.2 The structure of starch granules

Starch granules are semi-crystalline with a highly organized structure (Figure 2.1). After chemical or α -amylase treatment, starch granules show pronounced

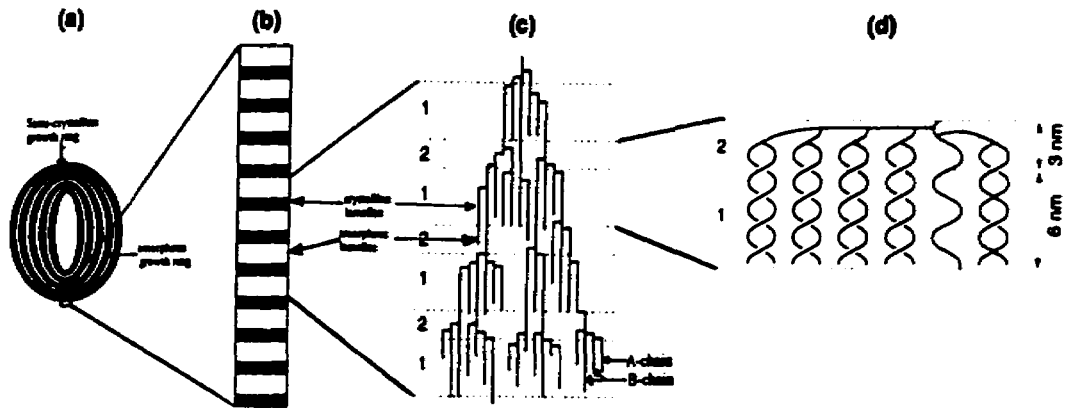


Figure 2.1. Schematic diagram of starch granule structure (adapted from Ball et al., 1998). (a) A single starch granule is composed of alternating concentric amorphous and semi-crystalline growth rings. (b) Expanded view of the internal structure. The semi-crystalline growth ring contains stacks of amorphous (white section) and crystalline (black section) lamellae. (c) The primary structure of an amylopectin molecule corresponding to amorphous and crystalline lamellae. (d) Part of the primary structure depicted in (c) is shown corresponding to the secondary structure proposed for amylopectin.

concentric growth rings under optical and electron microscopy (French, 1984). These growth rings are alternatively of semi-crystalline and amorphous composition (Yamaguchi et al., 1979; Jenkins and Donald, 1995). The semi-crystalline ring is composed of stacks of alternating crystalline and amorphous lamellae derived from amylopectin. Amylose molecules assuming single helical structures are packed into amorphous growth rings, which represent periodic growth because of daily fluctuations in carbohydrates available for starch deposition (French, 1984; Jenkins and Donald, 1995).

To form the semi-crystalline structure in starch granules, the A-chain and outer B-chain of amylopectin form double helices and associate into clusters, and the longer B-chains are involved in the formation of two or more clusters (Robin et al., 1974). These clusters are packed to produce a structure of alternating crystalline lamellae and amorphous lamellae (Figure 2.1b). Regions of double helices fall within the crystalline lamellae, while branch points lie in the amorphous lamellae (Figure 2.1c and d). The combined size of the crystalline plus amorphous lamellae is approximately 9 nm throughout the plant kingdom (Jenkins et al., 1993). Stacks of many alternating amorphous and crystalline lamella form the semi-crystalline growth rings which range from 120 to 400 nm in size (Jenkins et al., 1993). Amorphous growth rings have the similar size to and surround the semi-crystalline growth rings (Jenkins and Donald, 1995).

The precise structural role of amylose in starch granules is not clear. A large portion of the amylose seems to localize in amorphous rings, and some amylose may co-crystallize with amylopectin within the crystalline lamellae (Kasemsuwan and Jane,

1994). Amylose may also form complexes with lipid present within the starch granules, especially at the core of granules (Morrison, 1989). Jenkins and Donald (1995) found that increasing amylose contents in starch granules caused an apparent reduction of crystalline lamellae electron density, thus proposed that amylose may disrupt the packing of amylopectin double helices within the crystalline lamellae in two ways. First, amylose could co-crystallize with amylopectin, thus pulling the amylopectin chains out of the crystalline structure. Second, amylose chains may introduce disorder into the lamellar stacks by being oriented transverse to these stacks (Jenkins and Donald, 1995).

There are other non-starch components within starch granules, such as small amounts of lipid (0.1 – 1.0%) and proteins (0.05 – 0.5%). Proteins are either integral proteins, which are the enzymes for starch synthesis embedded in starch granules, or proteins absorbed on the surface of starch granules. Lipids are usually present associated with amylose in starch granules (Morrison and Gadan, 1987).

2.1.3 Biochemical aspects of starch granule biosynthesis

The starch synthetic pathway and the enzymes involved therein have been extensively reviewed previously (Preiss, 1991; Martin and Smith, 1995; Smith et al., 1997; Buléon et al., 1998; Preiss and Sivak, 1998; Båga et al., 1999). Here the generalized pathway of starch granule biosynthesis will be presented, and the relationship between the function of different starch synthetic enzymes and the composition and structure of starch granules will be reviewed.

2.1.3.1 The formation of ADPglucose

ADPglucose is the substrate for the synthesis of amylose and amylopectin (Preiss, 1991). ADPglucose pyrophosphorylases (AGPase) catalyze the synthesis of

ADPglucose from glucose-1-phosphate and ATP. AGPases are regulatory enzymes under allosteric control with inorganic phosphate and 3-phosphoglycerate as negative and positive effectors, respectively (Preiss and Sivak, 1998).

The importance of AGPase in starch synthesis results from extensive studies of AGPase mutants of different plant species. The deficiency in AGPase activity in the *sh2* (shrunk 2) mutants of maize causes a 70% reduction of starch yields (Tsai and Nelson, 1966), while the starch contents in the revertants of maize *sh2* mutations are increased by 15% because of the recovery of APGase activity (Giroux et al., 1996). In potato tubers, antisense RNA for AGPase reduces the activity of AGPase and starch content by 83 - 98.5% and 65 - 96%, respectively, as compared with wild-type potatoes (Müller-Rober et al., 1992). Stark et al. (1992) reported that expression of a mutated *E.coli* AGPase insensitive to allosteric regulation in potato tubers increased starch contents up to 60%. However, when a normal *E.coli* AGPase was expressed in potato tubers, starch synthesis was not affected significantly. Further, mutations in AGPase also alter the structure of starch granules. van den Koonhuyse et al (1996) showed that *sta1* mutants of *Chlamydomonas reinhardtii* defective for the large subunit of AGPase accumulate polysaccharides with a structure identical to that of transitory starch granules rather than reserve starch granules observed in wild-type. Therefore, all the studies with mutated or genetically modified AGPase provide strong evidence to support that AGPase is the major control point for starch synthesis in plants.

2.1.3.2 Initiation of the synthesis of starch molecules

The general concept about starch synthesis is that starch synthases transfer a glucosyl unit from ADPglucose to a non-reducing end of a pre-existing $\alpha(1, 4)$ -linked

glucan primer (Fekete et al., 1960). However, this raises the question of how starch synthesis is initiated. In bacteria and mammalian systems, a self-glycosylating protein, glycogenin, was found to be involved in the initiation of glycogen synthesis (Lomako et al., 1988). Glycogenin catalyzes the covalent attachment of a glucose residue on its tyrosine¹⁹⁴ and the formation of a nascent glycogen molecule up to eight glucose residues, which is used as a primer for glycogen synthesis. A similar mechanism was suggested for priming the synthesis of starch molecules in plants (Tandecarz et al., 1995). An enzyme, UDPglucose: protein transglucosylase (UDPG) from potato tubers and maize endosperm catalyzes the attachment of glucose from UDPglucose to its amino acid side chain. Subsequently, the glycosylated UDPG acts as a primer for starch synthesis (Moreno et al., 1986; Rothschild and Tandecarz, 1994). Purified UDPG from potato tubers is a homotetramer (made up of 38-kDa subunits) and could undergo self-glycosylation in an UDPglucose and Mn^{2+} -dependent reaction (Ardila and Tandecarz, 1992; Bocca et al., 1997).

2.1.3.3 The synthesis of amylopectin

It is well documented that amylopectin is responsible for the semi-crystalline structure of starch granules. The first line of evidence comes from the analysis of waxy starch granules. Despite the presence of very low concentration (< 1%) or the absence of amylose, waxy starch granules have the same structure as wild-type starch granules (Cooke and Gidley, 1992). Secondly, the polymodal distribution of the glucan chain lengths of amylopectin is the predominant determinant of the semi-crystalline structure in starch granules (Hizukuri, 1986). In maize and rice *sugary1* mutants, phytoglycogen is produced in the expense of amylopectin synthesis (Nakamura et al., 1997; Beatty et al.,

1999). With a different polymodal distribution of branch chain lengths from amylopectin, phytoglycogen becomes water soluble, and assumes a similar structure to glycogen rather than to starch granules (Shannon and Garwood, 1984). Therefore, the major aspects of starch granule biosynthesis would be explained through the elucidation of the mechanism underlying amylopectin synthesis.

The role of starch synthases

Starch synthases (SS) catalyze the elongation of α -(1, 4)-linked glucan chains by adding glucosyl moiety from ADPglucose to the non-reducing end of the pre-existing α -(1, 4)-linked glucan primer (Fekete et al., 1960). Multiple SS isoforms are involved in amylopectin synthesis, and are divided according to their amino acid sequences into three distinct classes: SSI, SSII and SSIII (Smith, 1999). Extensive studies of mutants defective for these isoforms have contributed greatly to the understanding of their involvement in the synthesis of amylopectin. In potato, SSII and SSIII account for about 10-15% (Edwards et al., 1995) and 80% (Marshall et al., 1996) of total soluble SS activity, respectively. Simultaneous reduction of SSII and SSIII activities through expression of antisense RNA causes serious disruption of starch granule morphology and severe alterations of amylopectin structure. Starch granules are distorted with deeply sunken-in centers, and clusters of small starch granules are produced (Edwards et al., 1999; Lloyd et al., 1999). Compared with the wild type, the amylopectin in SSIII/SSII antisense lines contains more glucan chains between dp (degree of polymerization) 7 and 13 (Edwards et al., 1999) and more very long chains, with fewer chains between dp 15 and 80 (Lloyd et al., 1999). In pea embryo, SSII contributes more than 60% of the total soluble SS activity (Denyer and Smith, 1992). Mutations at the pea *rug5* locus result in

the depletion of SSII activity and abnormal starch granule morphology. Amylopectin in the *rug5* mutants is enriched with short chains of dp 7 – 9 and very long chains, but is deficient in chains of intermediate length (dp 15 - 45) (Craig et al., 1998). In maize, the *Dul* gene codes for a soluble SS that is similar to potato SSIII and accounts for 20-30% of total soluble SS activity (Gao et al., 1998; Cao et al., 1999). The *dul* mutants display the appearance of some abnormally shaped starch granules, a more highly branched amylopectin, and the accumulation of 15% of starch as intermediate material (Wang et al., 1993). In *sta3* mutants of *C. reinhardtii*, where the activity of a 115 kD soluble SS is absent, the crystallinity of starch granules is altered. The amylopectin has a higher number of short chains (dp 2-7) and fewer chains of intermediate length, than that in the wild-type (Fontaine et al., 1993). All these studies demonstrate different SS isoforms may make distinct contributions to the synthesis of amylopectin.

The role of starch branching enzymes

The $\alpha(1, 6)$ linkages in amylopectin are produced by starch branching enzymes (SBE). SBE catalyze the hydrolysis of an $\alpha(1, 4)$ glucosidic bond and the subsequent attachment of the severed chain (containing the original non-reducing end) to a hydroxyl group of carbon-6 of a glucosyl unit in an $\alpha(1, 4)$ -linked glucan chain. Thus an $\alpha(1, 6)$ branch point is formed. Multiple isoforms of SBE have been found in many plant species, and can be grouped into SBE-I and SBE-II classes (Burton et al., 1995; Preiss and Sivak, 1996; Jobling et al., 1999). Maize SBEI, wheat SBEI, potato SBEI, pea SBEII and rice SBEI belong to SBE-I family, which has a higher affinity for amylose than amylopectin as an *in vitro* substrate and transfer long chains. The SBE-II class includes maize SBEIIa and SBEIIb, wheat SBEII, pea SBEI, rice SBEIII, which prefer

amylopectin as *in vitro* substrate and transfer shorter chains than those in the SBE-I class. Biochemical differences between the SBE-I and SBE-II classes suggest that the former participate *in vivo* in the synthesis of the long and intermediate length chains in amylopectin, while the latter is responsible for the production of the short chains (Preiss and Sivak, 1996).

Analysis of genetic mutants demonstrates the significance of the contribution of SBE to starch biosynthesis. The *ae* mutants of maize (Stinard et al., 1993) and rice (Mizuno et al., 1993) and *r* mutants of pea (Bhattacharyya et al., 1990) are defective for the activity of one SBE in the SBE-II class. The starch composition and concentration in these mutants are altered considerably: the amylopectin concentration is decreased from 70% to 30% with the appearance of an intermediate material which has longer branch lengths than normal amylopectin and a lower molecular weight than wild-type amylose. Starch content is reduced by up to 20% in *ae* mutant endosperm and up to 50% in *r* mutant embryos. The morphology of starch granules is also changed significantly by the loss of one isoform of the class II SBE. Granules from these mutants become small and irregular or deeply fissured (Shannon and Garwood, 1984). Antisense inhibition of the activity of SBE A, one enzyme in the SBE-II class of potato causes large structural alterations of amylopectin in potato tubers, which has more long chains (dp 23-60) and fewer short chains (dp 6-23) than the wild type (Jobling et al., 1999). In contrast, antisense inhibition of the activity of SBEI to very low levels in potato tubers results in no significant differences in amylopectin structure and granule morphology (Müller-Röber and Kossmann 1994; Flipse et al., 1996). However, a consistent alteration in starch physico-chemical properties is observed, indicating that a subtle modification of

amylopectin structure results from the antisense inhibition of SBEI activity in potato tubers (Safford et al., 1998).

The function of debranching enzymes

Although SBE catalyze the formation of $\alpha(1, 6)$ glycosidic bonds, their function can not produce the normal branching pattern, i.e. the spatial positioning of $\alpha(1, 6)$ glycosidic bonds, in amylopectin. Glycogen-like polysaccharides are synthesized *in vitro* with purified rice SBE (Nakamura, 1996). Expressed in *E.coli* defective for glycogen branching enzyme activity, maize SBE produces glycogen-like polyglucans instead of amylopectin (Guan et al., 1995).

Extensive studies of *sul* (*sugary1*) mutants in maize and rice (Pan and Nelson, 1984; James et al., 1995; Beatty et al., 1999; Nakamura et al., 1997; Kubo et al., 1999) and *C. reinhardtii sta7* mutants (Mouille et al., 1996) have established the key role of starch debranching enzymes (DBE), including isoamylase and pullulanase, in the determination of the branching pattern in amylopectin. Maize and rice *sul* mutants are deficient in both isoamylase and pullulanase activities. These mutants synthesize a highly-branched, water-soluble glucan called phytoglycogen in addition to amylopectin. In *C. reinhardtii sta7* mutants which lack one isoamylase of 88 kD, starch granules are replaced by phytoglycogen, (Mouille et al., 1996). Further, Zeeman et al. (1998) reported that an *Arabidopsis* mutant defective for isoamylase synthesizes both phytoglycogen and starch granules in leaf chloroplasts.

Despite the above evidence concerning DBE function in starch synthesis, there is some question as to whether DBE is directly involved in amylopectin synthesis. Ball et al. (1996) suggested that DBE is directly involved in amylopectin synthesis (Figure 2.2).

In contrast, Zeeman et al. (1998) proposed that DBE is not directly involved in amylopectin biosynthesis. Zeeman et al. (1998) suggested that the action of SS and SBE in amyloplast stroma resulted in the synthesis of soluble branched glucans, while the function of DBE was to prevent the accumulation of such soluble materials and direct glucan synthesis to the surface of granules. The reduction of DBE activity may prevent or reduce the hydrolysis of any $\alpha(1, 6)$ linkages in the soluble glucans, thus resulting in the accumulation of phytoglycogen and a consequent decrease in the amount of SS and SBE available for the synthesis of amylopectin at the granule surface.

A biochemical model for amylopectin synthesis

Neither the synthesis of glucans via starch synthetic enzymes *in vitro* nor the re-crystallization of solubilized amylopectin can produce a glucan with semi-crystalline structure (French, 1984). Glycogen has the same chemical composition as amylopectin, but assumes a tree-like structure and becomes a water-soluble polysaccharide rather than starch granules. Thus, the synthesis of amylopectin and its organization to form a granule is a highly integrated and regulated process. Ball et al. (1996) proposed a discontinuous synthesis model for amylopectin synthesis at the periphery of the growing starch granules (Figure 2.2). First, SS elongate short glucan chains from a previously synthesized and trimmed amorphous lamella at the periphery of starch granules. When these chains reach an appropriate length to accommodate the catalytic sites of SBE, branches are intensively and randomly generated through the action of SBE. Second, DBE trim down these unorganized branches, but do not have access to branch points close to the organized cluster zone. The action of DBE thus produces a region of short chains arising from branch points at the top of the cluster zone, from where the next

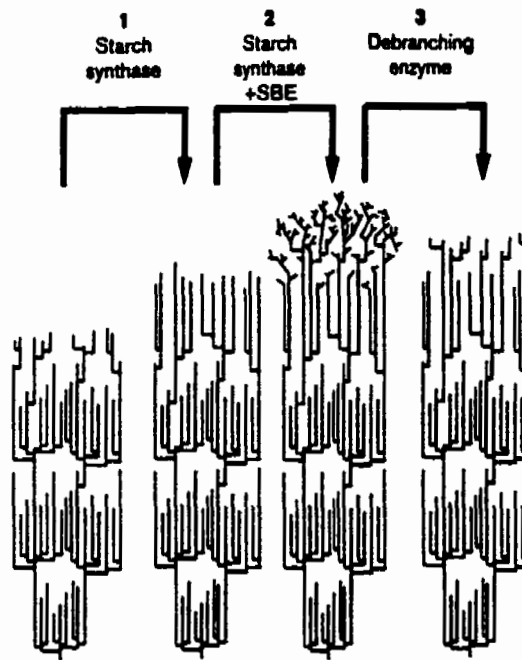


Figure 2.2. The discontinuous model for amylopectin synthesis as proposed by Ball et al. (1996) (Adapted from Smith, 1999).

round of elongation by SS is initiated. The discontinuous model explains the concerted actions of SS, SBE and DBE in the synthesis of amylopectin, the integration of amylopectin synthesis with its packing to form a starch granule, and the formation of an amylopectin cluster of constant size throughout the plant kingdom (Jenkins et al., 1993). However, there is still no *in vivo* experimental evidence for the validation of the discontinuous model.

2.1.3.4 The synthesis of amylose

Analysis of *wx* (waxy) maize (Shure et al., 1983), *wx* wheat (Nakamura et al., 1995), *wx* rice (Sano, 1984), *wx* barley (Hylton et al., 1996), *amf* (amylose-free) potato (Jacobsen et al., 1989), *lam* (low amylose) pea (Denyer et al., 1995b) and *sta2* *C. reinhardtii* (Delrue et al., 1992) establishes that starch granule-bound starch synthase I (GBSSI) is responsible for the synthesis of amylose component in starch granules. All of the above mutants are defective for GBSSI activity, and contain no or very little amylose in starch granules. However, these mutants accumulate normal amounts of starch granules with wild-type semi-crystalline structure (Cooke and Gidley, 1992), suggesting amylose synthesis starts after amylopectin is produced (Figure 2.3). This has been confirmed by *in vitro* amylose synthesis (Denyer et al., 1996; van de Wal et al., 1998).

van de Wal et al. (1998) proposed a pathway for amylose synthesis. GBSSI is tightly bound to an amylopectin matrix, and elongates the external chains of amylopectin. At critical lengths, these external chains are cleaved from amylopectin and become amylose (Figure 2.3). The cleavage may be catalyzed by granule-bound SBE, or a hydrolytic enzyme, or GBSSI itself. There is evidence to support this amylose synthesis pathway. First, *wx* mutants of rice (Takeda and Hizukuri, 1987) and *sta2*

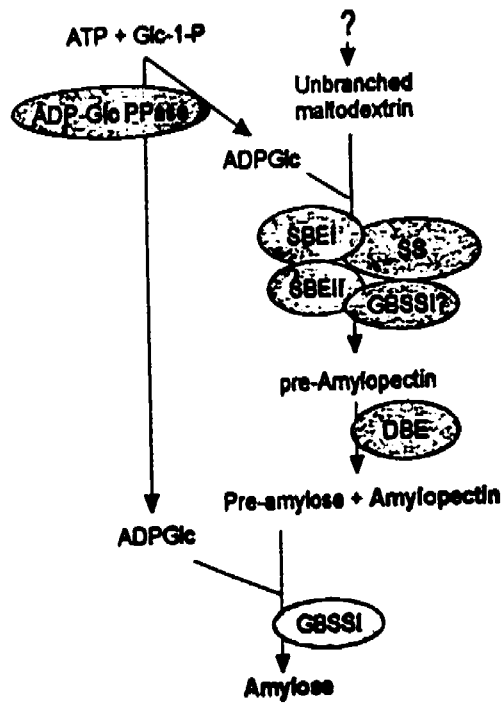


Figure 2.3. Schematic diagram of amylopectin and amylose synthesis as proposed by Preiss and Sivak (1996) (Adapted from Båga et al., 1999).

mutants of *C. reinhardtii* (Delrue et al., 1992) are not only deficient in amylose, but also in a fraction of amylopectin with very long glucan chains present in wild-type. Second, van de Wal et al. (1998) observed the extension of amylopectin external chains by GBSSI and subsequent cleavage into amylose *in vitro*, and the *in vitro* synthesized amylose was indistinguishable from native amylose in structure. Denyer et al. (1996) also demonstrated that GBSSI elongated glucan chains within amylopectin in isolated pea starch granules, and suggested both amylopectin and malto-oligosaccharides can act as precursors for amylose synthesis. Third, Tatge et al. (1999) reported that amylose synthesis occurs within the matrix of starch granules *in vivo*, and the space available in the matrix is an important determinant of the amylose content of storage starches.

2.1.4 Anatomical aspects of starch granule formation

Badenhuizen (1965, 1969) observed the initiation of starch granule formation in amyloplasts of developing maize endosperm. Before visible starch granules appear, a separate phase or coacervate droplet of amorphous starch molecules, proteins, lipid and amino acids accumulates in amyloplast. The coacervate droplets crystallize to form the nuclei or hilumla of developing starch granules when the accumulation of coacervate droplets reaches a critical amount. Wherever in the amyloplast starch granules have been crystallized, the accumulated coacervate droplets disappear. The crystallization may occur spontaneously, because the formation of a separate coacervate phase is the physico-chemical consequence of the incompatibility of aqueous solutions containing different high molecular weight polymers (French, 1984). In the amyloplast stroma, the high molecular weight polymers, such as amorphous starch molecules, proteins and lipids, crystallize to form starch granules.

It has been proposed that starch granules grow by apposition after the production of a small starch granule nucleus (Badenhuizen, 1965; Shannon et al., 1970). Badenhuizen (1965, 1969) reported that starch molecules were synthesized in the amyloplast stroma, and then deposited in the form of coacervate droplets on the periphery of developing starch granules. Shannon and co-workers (1970) exposed maize plants to $^{14}\text{CO}_2$ for starch synthesis, and found that ^{14}C was distributed throughout newly formed amylose and amylopectin molecules in starch granules, indicating that starch molecules are completely synthesized in the amyloplast stroma and then deposited on the starch granule surface. On the other hand, Ball et al. (1996) proposed that amylopectin was produced at the surface of the starch granule by starch synthase, starch branching enzyme and debranching enzymes, thus starch granules grew through extension of its peripheral starch molecules rather than apposition.

2.2. Starch Granules in Wheat Endosperm

2.2.1. Wheat endosperm has a bimodal starch granule size-distribution

At maturity, wheat endosperm contains two major types of starch granules: large A- and small B-type granules (Evers, 1971). A-type starch granules are disk-like or lenticular in shape, with an average diameter of 10-35 μm , whereas B-type starch granules are roughly spherical or polygonal in shape, ranging from one to ten μm in diameter (Buttrose, 1963; Evers, 1971). On average, A-type granules make up to 3% of the total number of starch granules in wheat endosperm, but contribute more than 70% of the total endosperm starch weight (Evers and Lindley, 1977). On the other hand, B-type granules account for over 90% of the total number of starch granules, but contribute

only about 25-30% of the total weight of starch in mature wheat endosperm (Evers, 1974; Morrison and Gadan, 1987; Stoddard, 1999).

Plotting the starch weight percentage or relative number of granules against granule diameter shows that wheat endosperm has a typical bimodal starch granule-size distribution curve, with the first peak as B-type granules and the second as A-type granules (Evers, 1973; Morrison, 1989; Seib, 1994). However, Bechtel et al. (1990, 1993) obtained a trimodal granule size-distribution by using quantitative image analysis coupled with dark field microscopy. In the trimodal distribution, in addition to A- and B-type starch granules, a C-type starch granule population is recognized. The C-type starch granules are less than five μm in size and compose 45.7%, and 3.4% of the total starch granule number and mass, respectively, at maturity. Reaker et al. (1998) reported a similar trimodal granule size-distribution in wheat endosperm.

2.2.2 The development of A- and B-type starch granules is different in wheat amyloplast

In wheat endosperm, amyloplasts are the sites for the synthesis of A- and B-type starch granules. However, the development of A-type starch granules is significantly different from that of B-type granules (Buttrose, 1963; Evers, 1971; Parker, 1985; Bechtel et al., 1990). Each amyloplast produces one A-type starch granule, which is initiated about four to five days-post-anthesis (DPA). The increase in the number of A-type granules stops about seven days later when cell divisions cease in the endosperm (Briarty et al., 1979). After that, the A-type granules do not grow in number, but they do increase in size to a final diameter from 10 μm to 35 μm at maturity (Dengate and Meredith, 1984). The formation of B-type granules is initiated in A-type amyloplasts

(amyloplasts containing one A-type starch granule) approximately 12-14 DPA (Parker, 1985; Bechtel et al., 1990; Seib, 1994). Both the number and size of B-type granules increase until grain maturity. However, the diameter of B-type starch granules is usually less than 10 μm (Morrison, 1989).

Based on the examination of the growth of starch granules from immature wheat kernels using a scanning electron microscope, Evers (1971) reported a developmental sequence for the formation of A-type starch granules. Before or at four DPA, initial, minute spherical granules are formed in amyloplast, and become the nuclei of A-type starch granules. The nuclei grow by progressive deposition of glucan polymers. Because the growth of the granular nucleus occurs from one side, the early form of the granular nucleus is partially surrounded by a lip-like structure, which eventually fuses as the lips advance toward each other in the equatorial plane. Subsequently, the nucleus is completely surrounded by a flange-like outgrowth. Further growth of the granular nucleus results in the formation of a large, lenticular A-type granule in an amyloplast at maturity.

Wheat B-type starch granules are found to originate from A-type amyloplasts (Buttrose, 1963; Parker, 1985). Buttrose (1963) observed that B-type granules appeared first between A-type granules and their enclosing amyloplast membrane. The membrane then protruded into the cytoplasm and constricted to release B-type granules. This observation was confirmed and extended by Parker (1985). He reported that before 12 DPA, each amyloplast contained one A-type granule. From 12-16 DPA, B-type granules are initiated within the stroma of some A-type amyloplasts, and they usually develop in the region of the groove and tubuli of A-type starch granules. Some A-type amyloplasts

produce narrow protrusions extending into the cytoplasm, where B-type granules develop to maturity and are subsequently released into cytoplasm.

2.2.3 Wheat A- and B-type starch granules have different chemical compositions

Wheat starch granules are composed of amylose, amylopectin, and small amounts of proteins and lipids which are entirely lysophospholipids (Ellis et al., 1998). Quantitative differences in these components were found between wheat A- and B-type starch granules (Morrison and Gadan, 1987; Seib, 1994; Reaker et al., 1998). B-type granules usually contain 2-3% less amylose, but a higher amount of lipid and granule-associated proteins than A-type granules. Further, amylose and amylopectin in wheat A- and B-type starch granules may have different structures. Takeda et al. (1999) analyzed the structure of amylose and amylopectin from barley A- and B-type starch granules. The amylose in B-type starch granules has much longer and a larger number of glucan chains than amylose in A-type granules. The amylopectin of B-type starch granules has a higher proportion of glucan chains of dp 6-9 and dp 16-19 and fewer glucan chains of dp 20-37 than A-type granule amylopectin. This results in the B-type starch granules having smaller amylopectin molecules than A-type.

The distribution of amylose, amylopectin, and lysophospholipids within A-type granules is significantly different from that within B-type granules, which results in the different enzyme digestibility between A-type and B-type granules (Morrison, 1989; Seib, 1994). The A-type granules are asymmetrical in the quantitative distribution of their components: the central region contains lower concentrations of amylose and lysophospholipids than the outside region. Digestion of A-type granules by amylases, such as salivary α -amylase and amyloglucosidase, produces holes on granule surfaces.

Amylases penetrate the granules through these holes and digest amorphous regions within granules, leaving pits all over the A-type granule surface and concentric shells of undigested crystalline material (Lorenz and Meredith, 1988). On the other hand, B-type granules are digested by surface erosion, and there is little evidence of pits on the granule surface and internal digestion, indicating that the distribution of starch components within the B-type granules is symmetrical (Morrison, 1989).

2.2.4 Wheat A- and B-type starch granules have different functional properties

The A- and B-type wheat starch granules differ not only in their chemical composition, but also in their functional properties, such as gelatinization, retrogradation behavior, pasting characteristics and baking properties (Kulp, 1973; Meredith, 1981; Eliasson and Karlsson, 1983; Soulaka and Morrison, 1985). Compared with A-type starch granules, B-type granules start to gelatinize at a lower temperature (T_0), but have a higher gelatinization peak temperature (T_p) and completion temperature (T_c) (Tester and Morrison, 1990). The most significant difference between the A- and B-type granules is that B-type starch granules have a much lower enthalpy than A-type granules (Soulaka and Morrison, 1985). The rheological properties of a starch gel are very sensitive to starch granule size. Higher B-type granule content in a gel increases the stiffness of the gel (Eliasson and Karlsson, 1983). The hot paste consistencies of both starch granules are similar, but compared with A-type, B-type granules are lower in hot paste stability and produce cold paste with lower consistency (Kulp, 1973). For baking applications, B-type granules have lower baking potential than A-type granules (Kulp, 1973); the breads prepared with blends containing a higher percentage of B-type granules are much lower in volume and poorer in quality than those made from blends with higher percentages of

A-type granules. Soulaka and Morrison (1985) also found that the highest specific loaf volume is obtained with 25-35% (weight/weight) B-type granules.

The differences in the chemical characteristics and functional properties of the A- and B-type wheat starch granules dictates their end uses in food and nonfood industries. Starch with predominantly B-type granules has been proposed as a fat substitute, because these granules are similar in size to lipid micelles (Jane et al., 1992). On the other hand, B-type starch granules are not desirable for the wet milling process, such as the manufacture of gluten and starch from wheat flour, because they are usually washed away, thus increasing the manufacturing cost (Stoddard, 1999). Starch with a high percentage of A-type granules has applications in the manufacture of biodegradable plastic film (Lim et al., 1992) and carbonless copy paper (Nachtergaele and Van Nuffer, 1989).

2.2.5 Starch granule size-distribution is genetically controlled

Starch granule size-distribution appears to be a trait under genetic control. Wheat, barley, rye, and triticale endosperm have bimodal starch granule size-distributions, while in other cereals, such as maize and rice, starch granule size-distribution is unimodal (French, 1984). However, mutations at the *s/x* locus result in barley endosperm with an apparent unimodal starch granule size-distribution (Schulman et al., 1994). Quantitative trait loci affecting the proportion of A-type starch granules in barley endosperm have been mapped on chromosome 2 (Borém et al., 1999). Dengate and Meredith (1984), Bechtel et al. (1993) and Stoddard (1999) investigated starch granule size-distribution in hundreds of wheat cultivars, and found that all the wheat genotypes have a biomodal starch granule size-distribution with slight modifications

caused by environmental effects. Genetic analysis by Stoddard (1998) demonstrated that the proportion of B-type starch granules in wheat endosperm is determined by additive genes with a narrow-sense heritability of approximately 0.59.

CHAPTER 3

SEPARATION AND CHARACTERIZATION OF A- AND B-TYPE STARCH GRANULES IN WHEAT ENDOSPERM

3.1 Abstract

Mature wheat (*Triticum aestivum* L.) endosperm contains two types of starch granules: large A-type and small B-type starch granules. Two methods, microsieving or centrifugal sedimentation through aqueous solutions of sucrose, maltose or Percoll were used to separate A- and B-type starch granules. Microsieving could not completely separate the two types of starch granules, while centrifuging through maltose and sucrose solutions gave a homogenous population of B-type starch granules only. Centrifuging through two Percoll solutions (70% and 100%, v/v) produced purified populations of both the A- and B-type starch granules. Analysis of starch granule size distribution in the purified A- and B-type granule populations and in the whole-starch granule population obtained directly from wheat endosperm confirmed that the purified A- and B-type starch granule populations represented their counterparts in mature wheat endosperm. Centrifugations through two Percoll concentrations were used to purify A- and B-type starch granule populations from six wheat cultivars. The amylose concentrations and gelatinization properties of these populations were analyzed. The A-type starch granules in the six cultivars contained higher amylose concentrations and had higher gelatinization enthalpies than did B-type starch granules. Although A- and B-type

starch granules started to gelatinize at a similar temperature, B-type starch granules had higher gelatinization peak and completion temperatures than did A-type starch granules.

3.2 Introduction

Wheat grain is one of the major cereals consumed by human beings. Starch is the predominant component of the wheat grain and constitutes two-thirds to three-quarters of the dry weight of a wheat kernel, depending on the cultivar (Hucl and Chibbar, 1996). At maturity, wheat endosperm contains two types of starch granules: large A- and small B-type starch granules (Evers, 1971). A-type starch granules are disk-like or lenticular in shape, with an average diameter of 10 - 35 μm , and contribute > 70% of the total weight, and about 3% of the total granule number, of endosperm starch. On the other hand, B-type starch granules are roughly spherical or polygonal in shape, ranging from 1 to 10 μm in diameter. These account for > 90% of the total granule number, but < 30% of the total weight of starch in wheat endosperm (Evers, 1973; Morrison and Gadan, 1987).

Wheat A- and B-type starch granules are reported to have significantly different chemical compositions and functional properties, such as amylose, amylopectin, lipid and protein concentrations, pasting characteristics and baking properties (Seib, 1994; Maningat and Seib, 1997). These differences result in the two starch granule types being utilized differently, both in food and nonfood uses. For example, starch with predominantly B-type starch granules can be used as a fat substitute (Lim et al., 1992), while starch with a high percentage of A-type starch granules has applications in the manufacture of biodegradable plastic film and carbonless copy paper (Nachtergaele and Nuffel, 1989). Thus, wheat cultivars with predominantly B- or A-type starch granules

would be very useful to the food and non-food industries. To develop these wheat cultivars, the biochemical characterization and ontogeny of A- and B-type starch granules during wheat kernel development must be understood. For this purpose, it is essential to develop methods that will efficiently separate the two types of wheat starch granules.

To separate wheat A- and B-type starch granules, several procedures, including microsieving (Evers, 1973), sedimentation in a water column (Morrison and Gadan, 1987) and elutriation (MacGregor, 1979) have been used. The separated starch granules were evaluated for chemical composition and functional properties. However, these procedures usually could not completely separate A- and B-type starch granules. Thus, the results from the subsequent characterization of A- and B-type starch granules should be used with caution. The objectives of the present study were: 1) to develop an efficient procedure to completely separate wheat A- and B-type starch granules; 2) to determine whether the separated A- and B-type starch granule populations could represent their counterparts in mature wheat endosperm; 3) to measure the amylose concentrations in A- and B-type starch granules, and 4) to study the gelatinization properties of A- and B-type starch granules.

3.3 Materials and Methods

3.3.1 Starch granule isolation

Wheat starch granules were isolated from mature seeds of *Triticum aestivum* cv. CDC Teal. Wheat grains (5.7 g) were steeped in 40 ml double distilled water (DDW) at 4 °C for 16 h. The softened seeds were degermed and ground in an autoclaved mortar

and pestle in 40 ml DDW. The slurry was filtered through four layers of cheesecloth to remove endosperm cell debris and centrifuged at 3500 x g for 5 min. The yellow gel-like layer on top of the packed white starch granule pellet was carefully removed. The starch granule pellet was then suspended in 5 ml of DDW, overlaid on 30 ml of 80% (w/v) cesium chloride, and centrifuged at 3500 x g for 5 min. The supernatant and cesium chloride with debris were discarded. The starch granule pellet, referred to as the whole starch granule population, was washed twice with 30 ml wash buffer (62.5 mM Tris-HCl, pH 6.8, 10 mM EDTA and 4% SDS), four times in DDW, and once with acetone. The starch granule pellet was then air dried and stored at -20°C .

3.3.2 Separation of A-type and B-type starch granules

The wheat starch preparation containing both A- and B-type granules was separated into the two types of granules by the following methods. 1) *Microsieving*. Wheat starch (0.5 g) was suspended in 200 ml of DDW. The starch suspension was filtered through two layers of nylon screen with a mesh diameter of 10 μm . The starch fraction retained by the screen was the A-type starch granule population, and that from the filtrate comprised the B-type starch granule population. 2) *Centrifugation through maltose (80%, w/v)*. A 5-ml volume of starch suspension in DDW (0.1 g/ml) was laid on the top of 10 ml 80% (w/v) maltose in a 15-ml glass tube and centrifuged at 10 x g for 10 min. The supernatant which contained B-type starch granules was removed to another tube. The pellet was washed two times in DDW, suspended in 5 ml DDW, and centrifuged four times in fresh 80% maltose solution. The starch pellet constituted the A-type starch granule population. The supernatants were pooled and centrifuged at 3500 x g for 5 min, and the resulting starch pellet comprised the B-type starch granule

population. Finally, A- and B-type starch granules were washed three times in DDW and once in acetone and then air-dried. 3) *Centrifugation through sucrose (80%, w/v)*. The separation procedure was the same as for maltose centrifugation except that maltose was replaced by 80% (w/v) sucrose. 4) *Centrifugation through Percoll (70 and 100%, v/v)*. A 5-ml of starch suspension (0.1 g/ml) was laid on the top of 10 ml 70% (v/v) Percoll solution (Pharmacia Biotech, Quebec) in a 15-ml centrifugation tube and centrifuged at 10 x g for 10 min. The supernatant, containing B-type starch granules, was removed to another tube. The pellet was washed twice in DDW, suspended in 5 ml DDW, and centrifuged three times in 70% (v/v) Percoll solution at 10 x g, 10 min. The pellet containing predominantly A-type starch granules was washed in DDW, suspended in 5 ml DDW, and purified to a homogeneous A-type starch granule population by three cycles of Percoll 100% (v/v) centrifugation (10 x g, 10 min in each cycle). Starch granules obtained from the pooled supernatants were considered as B-type starch granules. A- and B-type starch granules were washed and dried as described above for maltose centrifugation.

3.3.3 Starch granule size analysis

The sizes of starch granules from the purified A- and B-type starch granule populations and from the whole-starch granule population (directly from wheat endosperm) were analysed. Starch granules were suspended in 90% (v/v) ethanol. A drop of the starch suspension was spread on a microscope slide and air-dried. The slide was then placed on the stage of a light microscope (Leitz Laborluc K and D, Wetzlar, Germany). Images of starch granules were analyzed using an image analyzer equipped with image acquisition and processing software (BioQuant System IV, Image

Technology, New York). More than 1000 starch granules from each of the three starch granule populations (the purified A- and B-type and the whole-starch granule population) were analyzed. Starch granules were grouped according to diameter (Figure 3.1.B and 3.1.C), and the number of starch granules in each group was counted. Plotting the relative number of starch granules against granule diameters produced a starch granule size-distribution curve.

3.3.4 Determination of amylose concentration and starch gelatinization properties

Starch granules were isolated from the mature endosperm of five hexaploid wheat cultivars (*T. aestivum* cvs. CDC Teal, McKenzie, AC Karma, AC Crystal, and Fielder), and one tetraploid durum wheat (*T. turgidum* cv. Plenty). A- and B-type starch granules were separated using two Percoll solution centrifugations.

The total starch concentration of A- and B-type starch granules was analysed using the α -amylase/amyloglucosidase procedure (Megazyme total starch analysis kit, Megazyme, Wicklow, Ireland) (McCleary et al., 1994). A 100-mg starch sample (A- or B-type starch granules) was wetted with 0.2 ml of ethanol and completely dissolved by partial hydrolysis with thermostable α -amylase. Dextrins were then quantitatively hydrolysed to glucose by amyloglucosidase. The amount of glucose was determined, and the starch concentration was calculated on the basis of dry starch granules (0% moisture) as described by McCleary et al. (1994).

Amylose concentration in A- and B-type starch granules was analyzed as described by Gibson et al. (1997). A 25-mg starch sample was dissolved in one ml dimethyl sulfoxide solution by boiling for 15 min. Ethanol was added to precipitate starch and to remove lipid. The starch pellet was redissolved in dimethyl sulfoxide. An

aliquot (0.5 mL) was used to determine the total starch content. In another aliquot (1.0 mL), the amylopectin was precipitated with concanavalin A. The quantity of amylose remaining in the solution was determined after quantitative hydrolysis to glucose by amyloglucosidase/ α -amylase, and expressed as a proportion (%) of the total starch.

Gelatinization properties of A- and B-type starch granules were determined by differential scanning calorimeter (Mettler TA3000) as described by Abdel-Aal et al. (1997). A starch sample (4-5 mg) was loaded into a differential scanning calorimeter pan, and the requisite amount of water was added to give a volume ratio of 3:1 between water and starch. The sample was then heated at 10 $^{\circ}\text{C}/\text{min}$ and the transition temperatures recorded from a plot of heat flow versus temperature (30 – 100 $^{\circ}\text{C}$). The onset (T_0), peak (T_P) and completion (T_C) temperatures were taken from the curve and expressed in $^{\circ}\text{C}$. The enthalpy of gelatinization (ΔH , J/g) was calculated from measurement of the curve area using indium as the reference standard.

3.4 Results and Discussion

3.4.1 Separation of A-type and B-type starch granule populations

Wheat A- and B-type starch granules have significantly different sizes and rates of sedimentation in a water column (Seib, 1994), by which the two types of starch granules may be separated. In this study, microsieving separated A- and B-type starch granules according to their sizes, and centrifugation through maltose, sucrose and Percoll separated the two types of starch granules on the basis of their distinct rates of sedimentation in these solutions. Table 3.1 shows that microsieving could not completely separate A- and B-type starch granules, whereas centrifugation through

maltose or sucrose solution produced a purified B-type starch granule population, but could not give a homogeneous A-type starch granule population. However, centrifugation twice through Percoll (70 and 100%, v/v) completely separated the two types of starch granules (Figure 3.1.A).

In microsieving, many small B-type starch granules were attached to the surface of large A-type starch granules and were retained in the A-type starch granule population. Some large A-type starch granules passed through the nylon screen and, hence, were present with B-type starch granules in the filtrate. Eliasson and Karlsson (1983) found that the shape of starch granules also affected passage through microsieves. In this study, after microsieving, the A-type starch granule population contained about 67% of B-type starch granules, and the B-type starch granule population had > 20% of A-type starch granules (Table 3.1).

Sedimentation in a water column and elutriation separated A- and B-type starch granules based on the differences in sedimentation rate. However, because the density of water was low, the difference between sedimentation rates of A- and B-type starch granules in water was not large enough to separate the two types of starch granules. Therefore, neither sedimentation in a water column nor elutriation could completely separate A- and B-type starch granules (MacGregor, 1979; Eliasson and Karlsson, 1983). Eliasson and Karlsson (1983) reported that the A-type starch granule population from sedimentation in a water column contained about 20% (by number) B-type starch granules, while the B-type starch granule population contained about 30% (by weight) A-type starch granules.

Table 3.1. Efficiency of different methods to separate A- and B-type starch granule populations.

Method	B-type Granule Population (%)		A-type Granule Population (%)	
	A-type	B-type	A-type	B-type
Microsieving	^a 21.7	78.3	32.4	67.6
^b Maltose	0	100	92.0	8.0
^c Sucrose	0	100	89.6	10.4
^d Percoll	0	100	100	0

^aDiameter of starch granules was determined with micrometer under light microscope. Starch granules with diameter > 10 µm are A-type starch granules. Those < 10 µm are B-type starch granules. About 300 starch granules were analyzed for each method.

^bMaltose: single-density maltose (80%, w/v) centrifugation.

^cSucrose: single-density sucrose (80%, w/v) centrifugation.

^dPercoll: single-density two Percoll (70 and 100%, v/v), centrifugations.

We attempted to separate A- and B-type starch granule based on different sedimentation rates in maltose, sucrose, and Percoll solutions. Because maltose, sucrose, and Percoll solutions have higher densities than water, they may make the sedimentation rate difference between A- and B-type starch granules large enough to separate the two types of starch granules. Thus, in the present study, centrifugation through a single concentration of maltose or sucrose or through two concentrations of Percoll were employed. The B-type starch granule populations separated by these three methods contained 100% B-type starch granules (Table 3.1, Figure 3.1.A), while only centrifugation through two Percoll concentrations yielded a homogeneous A-type starch granule population (Table 3.1, Figure 3.1.A). A-type starch granule populations from maltose or sucrose centrifugation contained 8 –10 % B-type starch granules (Table 3.1). This is most likely due to the highly viscous solutions of 80% (w/v) maltose and sucrose, which made some B-type starch granules stick to the surface of A-type starch granules after centrifugation. Percoll is not sticky even at 100% (v/v) concentration. Thus, the separation of A- and B-type starch granules by centrifugation through two Percoll concentrations is complete and is not affected by the shape and size of starch granules.

3.4.2 Purified A- and B-type starch granules and their counterparts in mature wheat endosperm

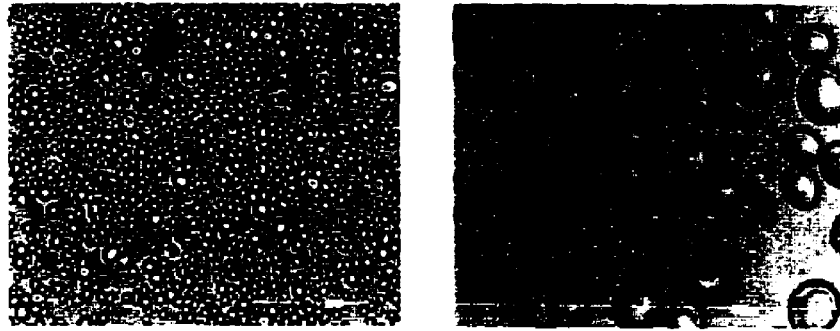
Although centrifugation through two Percoll concentrations produced purified A- and B-type starch granules, some starch granules (a mixture of A- and B-types) remained in the Percoll phase even after six cycles of centrifugation through two Percoll concentrations. This gave rise to the question whether the purified A- and B-type starch granules actually represented their counterparts in the whole-starch granule population.

Analysis and comparison of the starch granule size distributions in the whole starch granule population and the purified A- and B-type starch granule population were used to address this question.

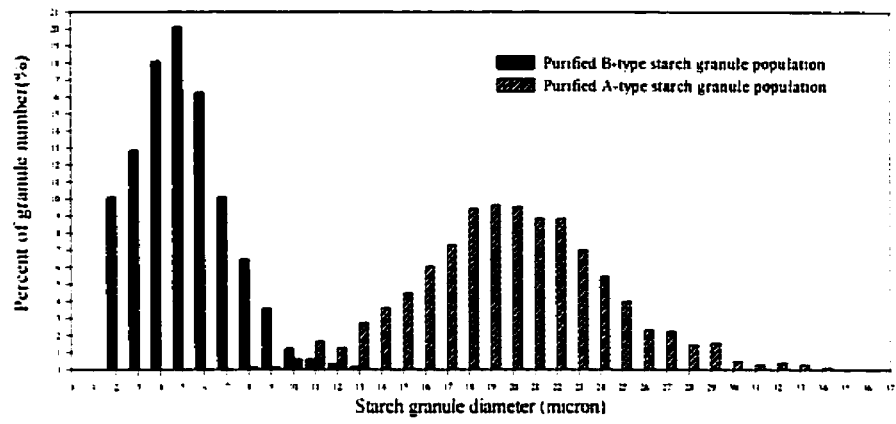
Starch granule sizes are usually measured by electrozone, image analysis, or laser-based time of transition methods (Maningat et al., 1996). Harrigan (1997) found that using image analysis to determine starch granule size could yield accurate and reproducible data. Thus, in this study, image analysis was used to determine wheat starch granule size distribution. Plotting the relative number of starch granules against granule diameters showed that the whole starch granule population had a typical bimodal granule size distribution (Figure 3.1.C). The first peak was at 4 – 6 μm and represented B-type starch granules. The second peak was at 16 – 19 μm and represented A-type starch granules. If > 50% of the granules in the purified B-type starch granule population are 4 – 6 μm in diameter and the diameters of most starch granules in the purified A-type starch granule population are in the range of the second peak, then the purified B- and A-type starch granule populations can represent their counterparts in a mature wheat endosperm. Figure 3.1.B showed that, in the purified B-type starch granule population, approximately 55% of the starch granules were 4 – 6 μm in diameter, and more than 60% of the starch granules in the purified A-type starch granule population were 16 – 22 μm in diameter. As expected, most starch granules retained in the Percoll phase were mixtures of A- and B-type starch granules, and ranged from 7 to 11 μm in diameter (data not shown). These starch granules accounted for the difference between A- and B-type starch granule peaks in the starch granule size-distribution curve of the whole-starch granule population (Figure 3.1.C).

Figure 3.1. Separation of A- and B-type starch granules. **A.** Purified large A-type starch granules (right) and small B-type starch granules (left) (Scale bar = 10 μm). **B.** Size-distribution curve of purified B-type starch granule populations; > 55% starch granules were 4 – 6 μm in diameter. In the purified A-type starch granule population, > 60% were 16 – 22 μm in diameter. **C.** Size-distribution curve of the whole-starch granule population. Two peaks appeared in the starch granule size-distribution of whole starch granule population. The first peak (4 –6 μm) represented B-type starch granules, and the second peak (16-19 μm) represented A-type starch granules.

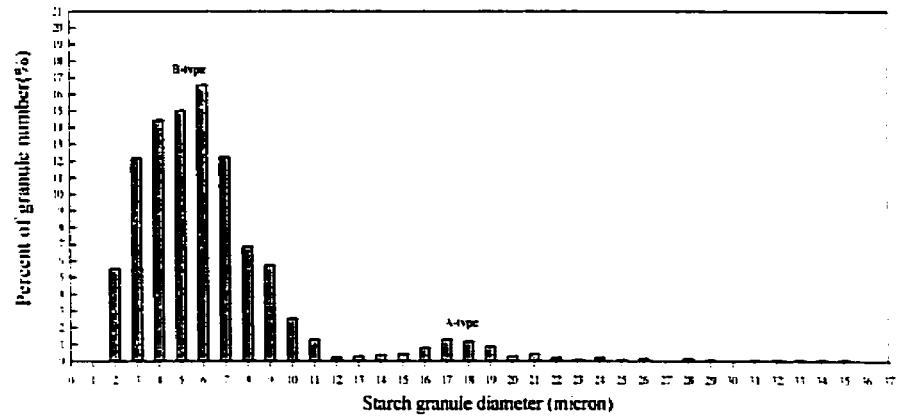
A



B



C



3.4.3 Amylose concentration and gelatinization properties of wheat A- and B-type starch granules

The characteristics of intact A- and B-type starch granules in mature wheat endosperm could be estimated by analysis of the chemical composition and gelatinization properties of the purified A- and B-type starch granules. In this study, amylose concentration, and gelatinization temperature and enthalpy were used to characterise A- and B-type starch granules.

Purified A- and B-type starch granules were obtained from six wheat cultivars. The data in Table 3.2 show that A- and B-type starch granules had similar total starch concentrations but significantly different amylose concentrations. A-type starch granules contained 30 – 36% amylose, while B-type starch granules contained 24 –27% amylose. This observation was consistent with those previously reported (Seib, 1994). However, in this study, the range of differences (3 - 10%) in amylose concentrations between A- and B-type starch granules was much larger than those published previously (2 -3%). This is most likely due to the increased purity of the A- and B-type starch granule populations. In previous studies (Eliasson and Karlsson, 1983; Souliaka and Morrison, 1985), A- and B-type starch granules were usually separated by sedimentation in a water column, therefore, the purity was lower than in the present study. The second reason may be that, in the current study, amylose was determined using the procedure described by Gibson et al. (1997), which is a more precise method than other commonly used methods, such as the measurements of the iodine binding capacity of amylose, or the separation of native amylose and amylopectin by size exclusion chromatography (Gibson et al., 1997).

Table 3.2. Amylose concentrations and gelatinization properties of purified wheat A- and B-type starch granules from six wheat cultivars

Cultivars		Amylose (%)	Starch (%)	Gelatinization by DSC			
				T _o (°C)	T _p (°C)	T _c (°C)	ΔH (J/g)
CDC Teal	A-type	35.5 ± 2.9*	92.1 ± 2.2	56.2	62.2	69.4	11.2
	B-type	26.4 ± 2.0	91.9 ± 1.9	55.4	63.4	71.6	9.2
McKenzie	A-type	34.9 ± 1.6	90.4 ± 2.4	52.2	60.2	70.3	10.2
	B-type	25.2 ± 0.5	90.5 ± 1.5	53.8	61.3	70.8	8.0
AC Karma	A-type	30.9 ± 0.6	91.3 ± 2.2	53.0	60.7	70.8	12.2
	B-type	27.2 ± 2.4	91.0 ± 1.9	54.4	62.4	73.2	9.0
AC Crystal	A-type	34.1 ± 2.9	91.3 ± 1.9	54.2	61.3	71.0	10.4
	B-type	26.8 ± 1.0	91.8 ± 1.6	55.0	62.3	72.6	8.3
Fielder	A-type	29.9 ± 2.0	90.8 ± 2.4	52.8	60.6	68.0	11.4
	B-type	24.6 ± 1.8	92.3 ± 2.2	53.4	61.8	68.4	10.0
Plenty	A-type	35.4 ± 2.8	91.1 ± 1.7	52.8	58.0	63.8	10.0
	B-type	27.4 ± 1.3	91.6 ± 1.5	52.2	59.6	69.6	8.8

*Mean ± SE. Each value was calculated from three repeats.

The gelatinization properties of wheat A- and B-type starch granules are usually measured by differential scanning calorimetry. It has been reported that A- and B-type starch granules have different gelatinization temperature regimes (Eliasson and Karlsson, 1983; Soulaka and Morrison 1985). Compared with A-type starch granules, B-type granules started gelatinization at a lower T_0 , but had higher T_p and T_C (Seib, 1994). In this study, B- and A-type starch granules started their gelatinization at a similar temperature, while the T_p and T_C temperatures of B-type starch granules were higher by 1-2 °C than those of A-type starch granules (Table 3.2). This is consistent with the observations of Eliasson and Karlsson (1983) and Soulaka and Morrison (1985), but differed from the results of Ghiasi et al. (1982), who reported that A- and B-type starch granules had similar gelatinization temperature regimes.

There are conflicting reports about the gelatinization enthalpy of wheat A- and B-type starch granules. Eliasson and Karlsson (1983) and Soulaka and Morrison (1985) reported that A-type starch granules had higher gelatinization enthalpy than B-type starch granules, while Stevens and Elton (1971) found the gelatinization enthalpies of wheat starch granules of different size classes to be similar. In contrast, Wootton and Bamunuarachchi (1979) reported that A-type starch granules had a lower gelatinization enthalpy than did B-type starch granules. From the measurements in this study, it was evident that the gelatinization enthalpy of A-type starch granules (10.0 - 12.2 J/g) was higher than that of B-type starch granules (8.0 - 10.0 J/g). This was consistent with the results from Eliasson and Karlsson (1983) and Soulaka and Morrison (1985). Imprecise measurements of the gelatinization enthalpy of A- and B-type starch granules were suggested as the cause of the conflicting reports (Soulaka and Morrison 1985).

In summary, starch granules from mature wheat endosperm have a typical bimodal granule size-distribution. The first peak represents small, B-type starch granules, while the second represents large, A-type granules. Centrifugation through two Percoll concentrations completely separated A- and B-type starch granules, which were confirmed to represent their counterparts in mature wheat endosperm. The experimental results in this study demonstrated that A- and B-type starch granules have significantly different chemical composition and gelatinization properties, indicating that they may have distinct applications in food and non-food industries.

CHAPTER 4

STARCH BRANCHING ENZYMES PREFERENTIALLY ASSOCIATED WITH A-TYPE STARCH GRANULES IN WHEAT ENDOSPERM

4.1 Abstract

Two starch granule-bound proteins (SGP), SGP-140 and SGP-145, were preferentially associated with A-type starch granules ($>10\ \mu\text{m}$) in developing and mature wheat (*Triticum aestivum* L.) kernels. Immunoblotting and N-terminal sequencing suggested that the two proteins were different variants of SBEIc, a 152 kDa isoform of wheat starch branching enzyme. Both SGP-140 and SGP-145 were localized to the endosperm starch granules, but were not found in the endosperm soluble fraction or pericarp starch granules younger than 15 days-post-anthesis (DPA). Small-size starch granules ($<10\ \mu\text{m}$) initiated before 15 DPA incorporated SGP-140 and SGP-145 throughout endosperm development and grew into full-size A-type starch granules ($>10\ \mu\text{m}$). In contrast, small-size starch granules harvested after 15 DPA contained only low amounts of SGP-140 and SGP-145 and developed mainly into B-type starch granules ($<10\ \mu\text{m}$). Polypeptides of similar mass and immunologically related to SGP-140 and/or SGP-145 were also preferentially incorporated into A-type starch granules of barley (*Hordeum vulgare* L.), rye (*Secale cereals* L.) and triticale (X *Triticosecale* Wittmack) endosperm, which like wheat endosperm have a bimodal starch granule size-distribution.

4.2 Introduction

Wheat, barley, rye and triticale mature endosperm contain large A-type and small B-type starch granules, thus showing a bimodal granule size distribution (French, 1984). In wheat, the large A-type starch granules are more than 10 μm in diameter and lenticular in shape, whereas B-type starch granules are less than 10 μm in diameter and roughly spherical (Evers, 1973). Wheat A- and B-type starch granules have significantly different chemical compositions and functional properties (Seib, 1994), and therefore, the development of wheat cultivars with predominantly A- or B-type starch granules would be of value to the food and non-food industries. To produce such wheat cultivars, it is necessary to understand the ontogeny of A- and B-type starch granules during wheat endosperm development.

Anatomical studies have revealed that A-type starch granules are initiated at about four to 14 DPA, during which the endosperm cells are actively dividing (Briarty et al. 1979; Parker 1985). On the other hand, B-type starch granules are initiated during the endosperm cell enlargement stage, which starts about 14 DPA and lasts until the wheat grain is mature (Briarty et al. 1979; Parker 1985). This differential production of the two types of granules suggests that the biosynthesis of A- and B-type starch granules in wheat endosperm is developmentally regulated.

Starch synthases (SS), starch branching enzymes (SBE) and starch debranching enzymes (DBE) participate in the biogenesis of plant starch granules (Ball et al. 1996; Preiss and Sivak, 1998). Each of these starch biosynthetic enzymes exists in multiple isoforms with different sub-cellular localization in developing wheat endosperm. Some isoforms are localized in endosperm soluble fractions or starch granules, and some are

present in both phases (Myers et al., 2000). Mutations inactivating any of these enzymes result in modification of starch structure, and sometimes also causes an altered starch granule morphology (Bhattacharyya et al., 1990; Mouille et al., 1996; Craig et al., 1998; Edwards et al., 1999). One enzyme suggested to have a role in determination of granule size in barley is the soluble SS. A mutation at the barley *s/x* locus results in lower SSI activity and a concomitant reduction in the size of A-type starch granules, thus giving the appearance of a uni-modal granule size-distribution (Schulman and Ahokas, 1990; Tyynelä and Schulman, 1993; Tyynelä et al., 1995). No mutant with altered starch granule size distribution, like *s/x* endosperm in barley, has been reported in wheat.

Among the SGP in wheat, several are likely to be actively involved in the production of amylose or amylopectin. The 60 kDa SGP, a granule-bound starch synthase (GBSSI), is required for synthesis of amylose (Shure et al., 1983), but GBSSI absence does not significantly affect granule size or structure (Fujita et al., 1998). On the other hand, absence of granule-bound SSII has been reported to cause deformation of large granules and production of starch with increased capacity to bind iodine (Yamamori, 1998). The major SGP in wheat starch range in size from 60 to 115 kDa (Rahman et al., 1995; Båga et al., 1999), but no significant difference in polypeptide profiles for these proteins extracted from A- and B-type starch granules has been found (Sulaiman and Morrison, 1990; Rahman et al., 1995). Recently, we isolated and characterized a cDNA encoding a novel SBEI, SBEIc, with predicted molecular mass of 152 kDa (Båga et al., 2000). SBEIc was found to be preferentially associated with starch granules of the wheat endosperm and corresponded to the 149 kDa SGP identified by Schofield and Greenwell (1987). Here, we show that most hexaploid wheat starches

contain two large SGP, SGP-140 (corresponding in mass to SBEIc) and SGP-145, that are preferentially incorporated into A-type starch granules. Polypeptides with masses similar to SGP-140 and SGP-145 were also present in other cereals showing a bimodal starch granule size-distribution. The possible involvement of SGP-140 and SGP-145 in the development of A-type starch granules is discussed.

4.3 Materials and Methods

4.3.1 Isolation of A-type and B-type starch granules

Starch granules were isolated from mature endosperm of five hexaploid wheat cultivars (*Triticum aestivum* L. cv. CDC Teal, McKenzie, AC Karma, AC Crystal, and Fielder), one tetraploid wheat (*Triticum turgidum* L. cv. Plenty) cultivar, barley (*Hordeum vulgare* L.), rye (*Secale cereals* L.), triticale (X *Triticosecale* Wittmack), rice (*Oryza sativa* L.), maize (*Zea mays* L.), canary seed (*Phalaris canariensis* L.) and potato (*Solanum tuberosum* L.) tubers as described (Peng et al. 1999). Pericarp and developing endosperm tissues were manually dissected from wheat (*Triticum aestivum* L. cv. CDC Teal) kernels and immediately placed in extraction buffer B (50 mM Tris-HCl, pH 7.5, 10 mM EDTA, 5 mM DTT, 10% glycerol, 0.1% (w/v) polyvinyl pyrrolidone held at 4°C. respectively. The pericarp fraction was washed three times with extraction buffer B to remove endosperm starch granules. Thereafter, the endosperm and pericarp fractions were homogenized with a mortar and pestle in three volumes of extraction buffer B and filtered through four layers of Miracloth (Calbiochem) to remove cell debris. The crude starch granules fraction was pelleted by centrifugation at 15,000 x g for 30 min and further purified as described (Peng, et al.,

1999). The endosperm starch granules were then separated into large-size (diameter >10 μm) and small-size (diameter <10 μm) fractions by centrifugation through two Percoll solutions (Peng et al., 1999). Image analysis of each granule fraction was performed as described (Peng et al., 1999).

4.3.2 Preparation of endosperm soluble fractions

The supernatant remaining from centrifugation of the homogenized endosperm (see above) constituted the endosperm soluble fraction. Protein concentration in the extract was determined using a dye-binding assay from Bio-Rad. For each endosperm fraction, the total amount of extracted soluble protein was determined.

4.3.3 SDS-PAGE and immunoblot analysis

To extract SGP, 50 mg starch granules were suspended in 350 μl extraction buffer A [62.5 mM Tris-HCl, pH 6.8, 10% (w/v) SDS, 5% (v/v) β -mercaptoethanol], boiled for 15 min, cooled to room temperature, and centrifuged at 15,000 $\times g$ for 20 min. SDS-PAGE analysis of SGP was done on 10% resolving gels (30:0.135) and proteins were visualized by Coomassie blue staining and/or silver staining (BIO-RAD). For immunoblot analysis, the gel-separated proteins were electrophoretically transferred at 4°C onto PVDF membranes (Millipore) using transfer buffer (25 mM Tris-HCl, pH 8.3, 192 mM Glycine and 20% methanol). Membranes were incubated, for 1 h in TBS buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl) containing 3% (w/v) bovine serum albumin, to block nonspecific binding sites. Antibodies, at a dilution of 1:4000 in TBS buffer, were then added to the blot and incubated for 4 h at room temperature. Following three washes in TBS buffer containing 0.05% Tween 20 and one wash in TBS buffer, membranes were incubated with alkaline phosphatase-conjugated goat anti-rabbit IgG

(Stratagene) at a dilution of 1:5000 for 1 h. Membranes were washed three times in TBS buffer containing 0.05% Tween 20, once in TBS buffer, and equilibrated in 20 mM Tris-HCl, pH 9.5, 100 mM NaCl, 5 mM MgCl₂. Immunoreactive bands were detected with 4-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate (Stratagene).

4.3.4 N-terminal sequencing of SGP-140 and SGP-145

SGP were extracted from 10 g A-type starch granules of CDC Teal and resolved on preparative SDS-PAGE gels. The migration of SGP-140 and SGP-145 was determined by silver staining a slice of the gel. The proteins were eluted from the unstained part of the gel using an electro-eluter (Model 422 Electro-Eluter. BIO-RAD) and elution buffer (25 mM Tris, 192 mM glycine, 0.1% SDS). The eluate was dialyzed for 8 h against 2 l of dialysis buffer (50 mM Tris-acetate, pH 6.8, 5 mM DTT), with one buffer change. The dialyzed solution was concentrated to 500 µl through ultrafiltration (Amicon 100), and 200 µl of the concentrate was loaded on a preparative SDS-PAGE gel. Gel-separated proteins were blotted on a PVDF membrane, as described above. SGP-140 and SGP-145 were identified by amido black staining and subjected to N-terminal sequencing using a gas-phase protein sequencer (Applied Biosystem Model 476A).

4.4 Results

4.4.1 Identification of granule-bound proteins preferentially associated with A-type starch granules in wheat endosperm

To compare SGP localized in A- and B-type starch granules, we purified the two granule fractions from wheat endosperm of six wheat cultivars using a method

previously reported (Peng et al., 1999). The extracted SGP were resolved by SDS-PAGE and visualized by silver staining. To quantitatively compare the different polypeptides in A- and B-type starch granules, the 60 kDa GBSSI was used as an internal standard for equal loading of proteins. The major SGP of 60, 80, 92, 100, 108 and 115 kDa. were present in similar concentrations in A- and B-type starch granules from all the cultivars tested (Fig 4.1), and no difference was observed among polypeptides with molecular masses lower than 60 kDa (data not shown). These results were consistent with previous studies that reported almost identical polypeptide profiles for wheat A- and B-type starch granules (Sulaiman and Morrison, 1990; Rahman et al., 1995).

In addition to the major SGP, it was recently found that a novel SBEI isoform, SBEIc, migrating as a 140 kDa polypeptide on SDS-PAGE gels, was associated with starch granules of the cultivar Fielder (Båga et al., 2000). In this study, we observed that A-type starch granules of all wheat cultivars tested contained a polypeptide co-migrating with SBEIc (Fig 4.1). A slightly larger polypeptide, with an apparent molecular mass of 145 kDa, was also present in A-type starch granules of all cultivars except Fielder (Fig 4.1). Analysis of B-type starch granules from the six wheat cultivars showed a much lower abundance of the 140 and 145 kDa polypeptides as compared to the A-type granules (Fig 4.1). In the B-type granules of the cultivar Fielder, only the 140 kDa band was observed, as was found for the A-type granules of this cultivar. The lower abundance of the 140 and 145 kDa polypeptides in B-type starch granules suggested that SGP-140 and SGP-145 are incorporated into B-type granules, albeit with much lower efficiency than into A-type granules. Alternatively, the B-type granules do not contain SGP-140 and SGP-145, and the weak bands we observed resulted from contamination of B-type starch granules

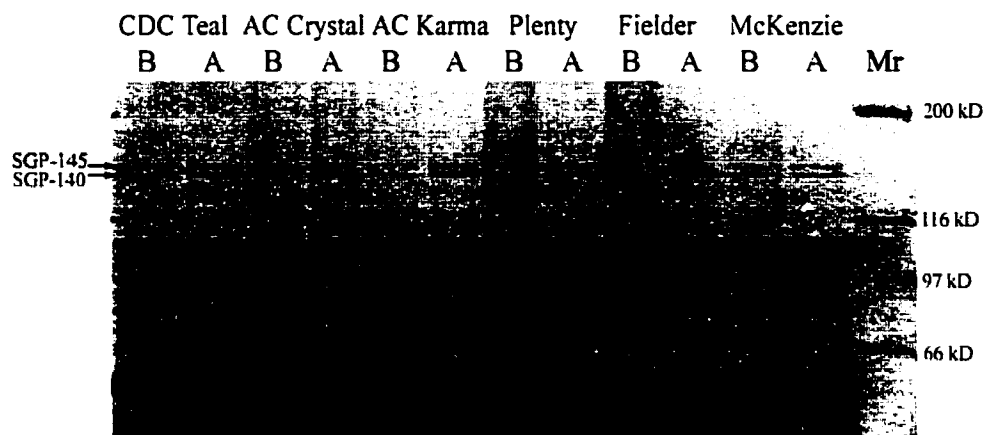


Figure 4.1. SDS-PAGE analysis of SGP extracted from wheat A- and B-type starch granules. Each lane was loaded with protein extract from 5 mg A- and B-type starch granules of five hexaploid and one tetraploid (Plenty) cultivar. Separated proteins were visualized by silver staining and migration of protein molecular weight markers (Mr) is indicated to the right.

with some small-size A-type starch granules. Nevertheless, the conclusion from our data was that the SGP-140 and SGP-145 were preferentially associated with A-type starch granules.

4.4.2 SGP-140 and SGP-145 are preferentially incorporated into A-type starch granules throughout endosperm development

In developing wheat endosperm, A-type starch granules are initiated at about four to 14 DPA, whereas B-type granules are formed after 14 DPA (Briarty et al., 1979; Parker, 1985). After initiation, both granule types continue to grow until maturity of the endosperm (Morrison and Gadan, 1987). An image analysis of purified large-size and small-size starch granule fractions from developing endosperm of the cultivar CDC Teal showed that the growth of small starch granules formed before and after 15 DPA was significantly different (Fig 4.2). Prior to 15 DPA, the newly formed small starch granules grew rapidly in size to become large-size ($>10\text{ }\mu\text{m}$) starch granules (Fig 4.2A). During the time period eight to 15 DPA, large-size starch granules accounted for more than 70% of total endosperm starch granules (Fig 4.2B). Small-size starch granules formed after 15 DPA increased rapidly in number until maturity (from 25% to 94%), but they grew very slowly and only reached diameters less than $10\text{ }\mu\text{m}$ (Figs. 2A and 2B).

The preferential incorporation of SGP-140 and SGP-145 into A-type granules could be explained by expression of these polypeptides only during the first 15 DPA. To test this hypothesis, we analyzed the protein profiles of large-size and small-size granules isolated at different DPA (Fig 4.3). The large-size ($>10\text{ }\mu\text{m}$) A-type starch granules were found to show no variation in SGP-140 and SGP-145 concentration during development. Small-size starch granules ($<10\text{ }\mu\text{m}$ in diameter) formed before 15 DPA, which were of

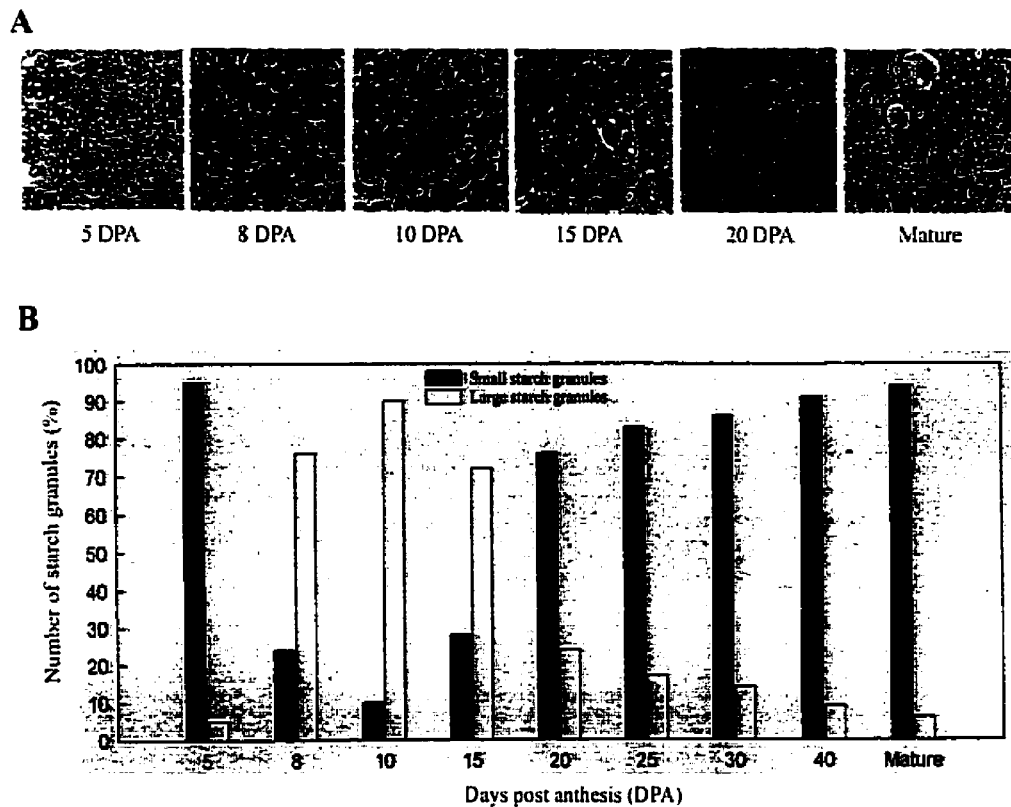


Figure 4.2. Analysis of starch granule size distribution in wheat endosperm. **A.** Light microscopic pictures (500 x) of total starch granules harvested at different stages of endosperm development of the hexaploid wheat cultivar CDC Teal. **B.** Histogram of small-size (< 10 μm) and large-size (> 10 μm) granule size distribution during wheat endosperm development.

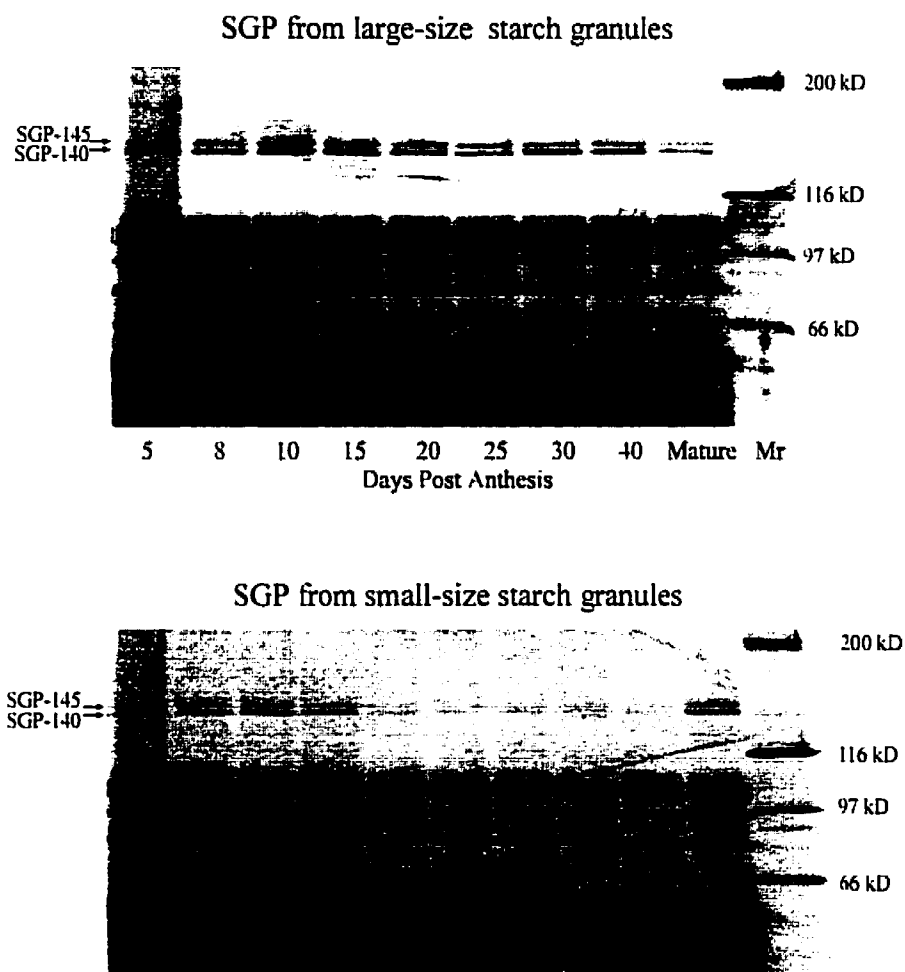


Figure 4.3. SDS-PAGE analysis of SGP extracted from small-size ($< 10 \mu\text{m}$) and large-size ($> 10 \mu\text{m}$) starch granules of the hexaploid wheat cultivar CDC Teal. Samples of SGP from 5 mg starch granules were from different stages of wheat endosperm development as indicated. Gel-separated proteins were visualized by silver staining and migration of protein molecular weight marker (Mr) is indicated to the right.

the A-type, were also found to contain SGP-140 and SGP-145 at about the same concentration as in large-size granules. On the other hand, small-size starch granules harvested after 15 DPA, which are mainly of B-type, showed very low presence of SGP-140 and SGP-145. The analyses demonstrated no significant variation in concentration of the other major granule-bound polypeptides (60, 80, 92, 100, 108 and 115 kDa) for both small-size and large-size starch granules throughout endosperm development. In the cultivar CDC Teal, most of the A-type granule growth occurred after 15 DPA, when about 65% (w/w) of the starch in A-type granules was synthesized. Thus, the constant abundance of SGP-140 and SGP-145 in A-type granules strongly suggested that the two proteins were continuously incorporated into A-type granules throughout endosperm development.

4.4.3 SGP-140 and SGP-145 are immunologically related to SBEI

To confirm the identity of SGP-140 as a SBEI isoform in the cultivar CDC Teal and to possibly identify SGP-145, immunoblots of SGP from A- and B-type starch granules were reacted with polyclonal antibodies raised against wheat SBEI, SBEII, SSI, SSII and GBSSI, respectively (Fig 4.4). The major polypeptides of 60 kDa (GBSSI), 80 kDa (SSI), 92 kDa (SBEII) and 100 to 115 kDa (SSII), were recognized by their respective antibodies, as expected, with no difference in intensity between A-type and B-type granules (Fig 4.4). Among the five antibodies tested, only the wheat SBEI antibodies reacted with SGP-140 and were also found to recognize SGP-145. A weaker interaction between the SBEI antibodies and a protein co-migrating with SBEII and proteins of approximately 63 kDa were also seen. Similar to the analysis of SGP-140 and

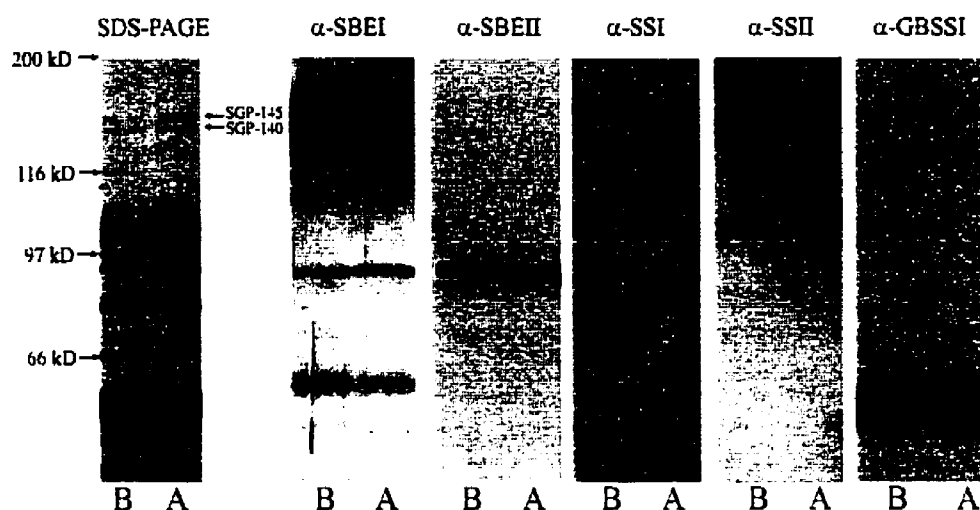


Figure 4.4. Immunoblot analysis of extracted SGP from wheat A- and B-type starch granules. Each lane was loaded with SGP extracted from 2 mg A- and B-type starch granules harvested from mature endosperm of the hexaploid wheat cultivar CDC Teal. To the left is shown SGP separated by SDS-PAGE and visualized by silver staining. To the right is shown immunoblot analyses of gel-separated SGP using polyclonal antisera prepared against different wheat starch biosynthetic enzymes as indicated.

SGP-145 by SDS-PAGE, the immunoreactive bands were strong in A-type, but weak in B-type starch granules (Fig 4.4).

To compare SGP-140 and SGP-145, both protein bands were purified from SDS-PAGE gels and subjected to direct amino acid sequencing. The sequence information from this analysis suggested variation in amino acid sequence as indicated in Table 4.1. This is likely due to presence of several polypeptides that differ slightly in sequence within the same protein band as suggested by reverse transcription PCR analysis (Båga et al., 2000). Nevertheless, alignment of the determined N-terminal sequences of the SGP-140 and SGP-145 with those predicted for SBEIc and wSBEI-D2 revealed striking similarities, thus suggesting that all four polypeptides were closely related (Table 4.1). A lower level of similarity was noted to the predicted N-terminal sequence for the wheat 87 kDa SBEIb isoform (Repellin et al., 1997). Since the molecular masses of SGP-140 and SGP-145 were reasonably close to that of SBEIc (152 kDa) predicted from *SbeIc* cDNA, the data suggested that SGP-140 and SGP-145 were isoforms of SBEIc.

4.4.4 SGP-140 and SGP-145 are endosperm starch granule-bound SBEI

Sub-cellular localization of starch biosynthetic enzymes is of importance for understanding their function. To localize SGP-140 and SGP-145 in the developing kernels, SGP from pericarp and endosperm starch granules, and the endosperm soluble fraction were prepared from developing wheat kernels, and analyzed by SDS-PAGE and immunoblotting. The results of these analyses confirmed that SGP-140 and SGP-145 were present within the endosperm starch granules, but could not be found in the endosperm soluble fraction (Fig 4.5). Nor were SGP-140 and SGP-145 observed in pericarp starch granules harvested from 5 to 10 DPA, but could be seen as two very faint

Table 4.1. Alignment of SGP-140 and SGP-145 N-terminal sequences to those predicted for wheat endosperm SBEI and SBEI-like proteins.

Polypeptide	Sequence
SGP-140	K/H/A I/V N G Y G R D R E R M Y D R
SGP-145	Q I I G Y G S D H L E M Y D E
SBEIc (152 kDa)	A N G Y G S D H L E M Y D E
wSBEI-D2 (87 kDa)	I I G Y G S D H L E I Y D E
SBEIb (87 kDa)	Y T A I A E D G V G D E I Y D E

Wheat wSBEI-D2 is a SBEI-like protein predicted to be produced in wheat endosperm (Rahman et al. 1997), SBEIc is deduced from a wheat endosperm transcript (Båga et al. accompanied manuscript) and SBEIb is deduced N-terminal sequence of 87kDa SBEI expressed in wheat endosperm (Repellin et al. 1997). Identical amino acids are highlighted.

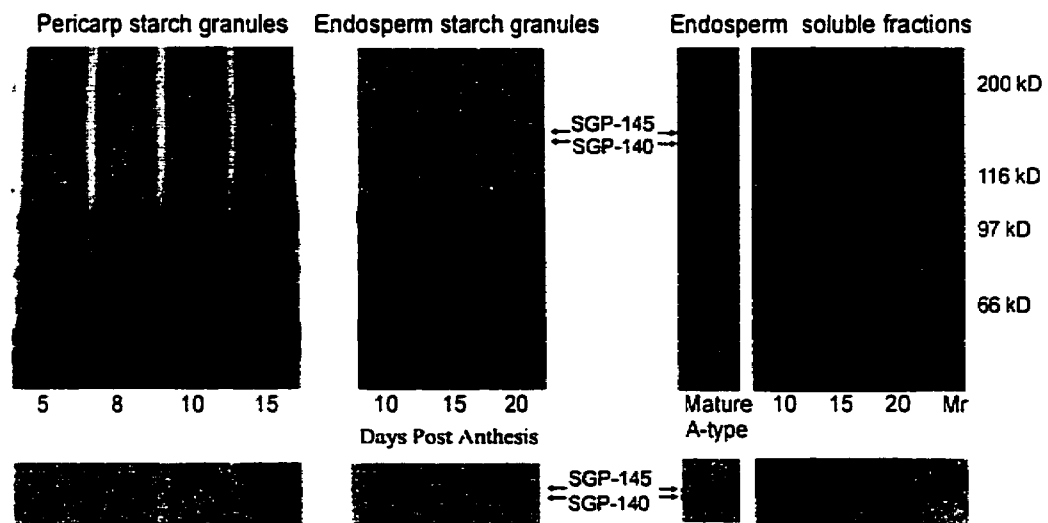


Figure 4.5. Sub-cellular localization of SGP-140 and SGP-145 in immature wheat kernels. SDS-PAGE analysis of endosperm soluble fractions and SGP extracted from CDC Teal pericarp and endosperm starch granules. Samples were prepared from endosperm of different developmental stages as indicated. Each lane was loaded with SGP from 5 mg starch granules, or 280 (10 DPA), 250 (15 DPA) or 250 (20 DPA) μ g protein from endosperm soluble fractions. These soluble protein amounts corresponded to the same amount of endosperm containing 5 mg starch. Gel-separated proteins were visualized by silver staining (pericarp and endosperm starch analysis) or Coomassie blue staining (soluble endosperm analysis). Migration of molecular weight marker (Mr) is shown to the right. Below is shown immunoreactive bands formed between gel-separated SGP-140 and SGP-145 and wheat SBEI antibodies.

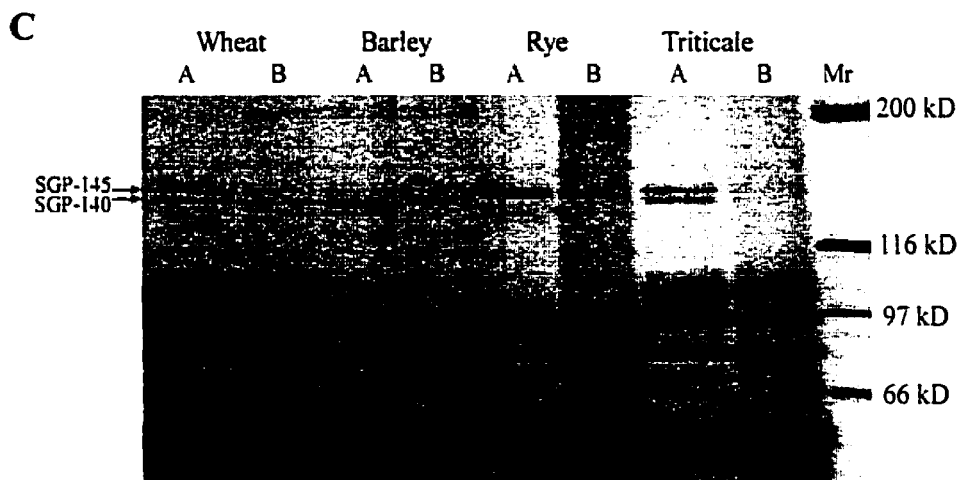
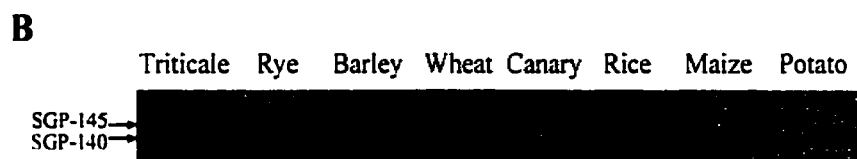
bands in pericarp granules of 15 DPA (Fig 4.5). Since pericarp from kernels older than 15 DPA was rather difficult to separate from the endosperm, it is possible that the two faint bands seen in 15 DPA pericarp sample originated from some endosperm starch granules mixed with the pericarp starch granules.

4.4.5 SGP-140 and/or SGP-145 exist in plant species known to produce A- and B-type starch granules

We extended the study of starch granule proteins to other plants than wheat to determine if any association could be found between the size-distribution of granules produced and the presence of SGP-140 and SGP-145 homologs. This study included starches from plants with bimodal (rye, barley and triticale) and unimodal (rice, maize, potato, canary seed) starch granule size-distribution (French, 1984). SDS-PAGE analysis of extracted SGP from triticale, barley and rye starch granules revealed one (barley and rye) or two protein bands (triticale) with similar relative mobility to SGP-140 and SGP-145 of wheat (Fig 4.6A). These protein bands were also found to react with SBEI antibodies (Fig 4.6B), and thus appeared to be SGP-140 and SGP-145 homologs. Analysis of SGP from starch granules of canary seed, rice, maize and potato tubers did not reveal any presence of SGP similar in size to SGP140 and SGP-145 and reacting with SBEI antibodies (Fig 4.6A and 4.6B). Thus, it appeared that proteins similar to SGP-140 and SGP-145 were only present in cereal starches with bimodal granule size-distribution.

To determine if the SGP-140 and SGP-145 counterparts in triticale, barley and rye were, like in wheat, preferentially associated with A-type starch granules, the A- and B-type starch granules from these cereals were analyzed. Similar to wheat endosperm

Figure 4.6. Analysis of SGP in starches from various plant sources. **A.** SDS-PAGE analysis of SGP extracted from 5 mg starch of: A-type starch granules from endosperm of triticale, wheat, barley and rye; total starch from endosperm of canary seed, rice and maize; and potato tubers. Proteins were visualized by silver staining. Migration of molecular weight marker (M_r) is shown to the right. **B.** Immunoblot analysis of gel-separated proteins shown above. Immunoreactive bands obtained from interaction between wheat SBEI polyclonal antibodies and SGP-140 and SGP-145 are indicated. **C.** SDS-PAGE analysis of extracted SGP from 5 mg A- and B-type starches isolated from wheat, barley, rye and triticale endosperm. Proteins were visualized by silver staining. Migration of molecular weight marker (M_r) is shown to the right.



starch, the SGP-140 and SGP-145 homologs were abundant in A-type starch granules, but very scarce in B-type starch granules (Fig 4.6C).

4.5 Discussion

The biogenesis of starch granules in plant amyloplasts involves two successive steps: the formation of small starch granule nuclei, and the production of mature granules by apposition of starch molecules onto the nuclei (Badenhuizen, 1965; Shannon et al., 1970). Thus, the biosynthesis of A- and B-type starch granules in developing wheat endosperm could be regulated at two stages. The first stage would be during the formation of the starch granule nuclei. Previous reports, and our data, strongly suggest that one peak of granule nucleus formation occurs before 15 DPA, and another occurs after 15 DPA. The second stage of regulation could be during the development of the nuclei into A- and B-type granules. During this stage, the A-type granules are able to grow larger than 10 μm in diameter, and B-type granules lack this ability.

Our results show that SGP-140 and SGP-145 are preferentially found on both small-size and large-size A-type granules (Fig 4.3). No reduction was noted in SGP-140 and SGP-145 concentrations in large granules harvested after 15 DPA (Fig 4.1), a developmental stage when most of the A-type granule starch is being produced. This argued against SGP-140 and SGP-145 being incorporated at a specific stage of A-type granule development, but rather, continuously targeted to A-type granules, even when B-type granules are produced. Since SGP-140 and SGP-145 did not accumulate in the soluble phase of the endosperm, these proteins must be actively produced both before and after 15 DPA. This was also indicated by RNA analysis of SGP-140 expression

during kernel development, which showed only a small reduction in transcript levels after 15 DPA, as compared to before 15 DPA (Båga et al., 2000).

In developing wheat endosperm, only one A-type starch granule is produced in each amyloplast (A-type amyloplast) from four to 14 DPA, a stage when the endosperm cells are dividing (Briarty et al., 1979). During the cell expansion stage of endosperm development (about 15 DPA to maturity), the B-type starch granules appear in the protrusions extending from A-type amyloplast (Parker, 1985). The formation of A- and B-type starch granules at different locations could be the reason for preferential localization of SGP-140 and SGP-145 to A-type starch granules.

SGP-140 and SGP-145 were also found to be associated with A-type starch granules in the endosperm of barley, rye, and triticale, but no presence of similar polypeptides could be detected in the potato starch granules, which are also relatively large in size. These results suggest that SGP-140 and SGP-145 homologs are not generally associated with large starch granules in plants. Furthermore, we were unable to detect SGP-140 and SGP-145 in starch granules from endosperm of canary seed, rice and maize, which like potato starch granules, have a unimodal size distribution. These data suggest that the presence of SGP-140 and SGP-145 was related to the presence of A- and B-type starch granules in wheat, rye, barley and triticale.

The amino-terminal sequencing and immunoblotting of SGP-140 and SGP-145 produced in the wheat cultivar CDC Teal, strongly suggested that these polypeptides are isoforms of SBEIc identified in the wheat cultivar Fielder (Båga et al., 2000). Both SGP-140 and SGP-145 were present in the endosperm starch granules and absent in the soluble fraction. Thus, SGP-140 and SGP-145 differ in sub-cellular localization from the

main isoforms of SBEI (87 to 88 kDa), which are primarily found in the soluble fraction of the endosperm (Morell et al., 1997). The different locations of the 87 to 88 kDa SBEI and the much larger SGP-140 and SGP-145 may imply that the two classes of SBEI have different activities and functions in the wheat endosperm. It is possible that the 87 to 88 kDa SBEI are functional in the synthesis of amylopectin in the endosperm soluble fraction, but become inactive when trapped within starch granules, like their counterparts in pea and maize (Denyer et al., 1993; Mu-Forster et al., 1996). In contrast, SGP-140 and SGP-145 may, like the exclusively granule-bound GBSSI, be primarily active only on polymers of the starch granule. Thus, it is conceivable that the amylopectin produced by the soluble 87-88 kDa SBEI and SGP-140 and SGP-145 may differ in structure.

Since SS, SBE and DBE participate in the biogenesis of plant starch granules, we speculate that one or several isoforms of these enzymes are involved in the regulation of initiation and size growth of A- to B-type starch granules in the developing wheat endosperm. Identification of the SGP-140 (SBEIc) and SGP-145 and their occurrence coinciding with A-type starch granules suggest that these proteins may play some role in the growth of small-sized A-type into full-sized A-type starch granules. This role may be to regulate the amount and/or structure of amylopectin molecules formed in the small-size A-type starch granules, which allows the A-type granules to expand to a larger extent than the B-type granules. However, to test this theory further, characterization of SGP-140 and SGP-145 isoforms and their action on glucan polymers is needed.

CHAPTER 5.
MOLECULAR CLONING AND CHARACTERIZATION OF STARCH
SYNTHASE I IN WHEAT (*Triticum aestivum* L.) KERNELS.

5.1 Abstract

Two full-length cDNA, *wSsI*-I and *wSsI*-II, encoding wheat starch synthase I (wSSI; E.C. 2.4.1.21) were isolated from a wheat endosperm cDNA library. DNA sequence analysis suggested that the isolated cDNA clones code for SSI. The translated amino acid sequences for *wSsI*-I and *wSsI*-II predicted primary products of 647 amino acids, which shared 99.2% amino acid sequence identity, and a pre-protein with a molecular mass of 71 kDa. A 41 amino acid transit peptide was postulated to cleave from both the pre-proteins to give a mature polypeptide of 67 kDa. Expression of the *wSsI* cDNA, in a glycogen synthase deficient *E.coli* strain RH98, complemented its glycogen synthase deficiency, thus demonstrating it encoded a functional starch synthase. RNA gel-blot analysis revealed that *wSsI* transcripts were detected in leaf, stem, root, pre-anthesis floret, ovary and pollen. During endosperm development, a higher level of accumulation of *wSsI* transcripts was observed at five - 10 days-post-anthesis (DPA) than at 15 - 25 DPA. The wSSI is localized to both the endosperm soluble fraction and starch granules. However, during endosperm development, wSSI was present at a constant level in starch granules, but in endosperm soluble fractions, it was not detected at five DPA, and had highest concentrations from 10 to 15 DPA. On a per kernel basis, wSSI had similar concentrations from 15 to 25 DPA, but starch

granules had considerably higher concentrations than did endosperm soluble fractions. This uneven distribution of wSSI was more prominent at the later stages of endosperm development.

5.2 Introduction

Starch, the major storage compound in cereal endosperm, is composed of two distinct types of glucose polymers, amylose and amylopectin. Starch synthases (SS; E.C. 2.4.1.21) catalyze the elongation of $\alpha(1-4)$ linked glucan chains in amylose and amylopectin by the addition of the glucosyl moiety from adenosine diphosphate glucose (ADP-Glc). Plant storage organs contain multiple isoforms of SS. Based on their primary amino acid sequence similarities, the SS isoforms identified so far can be grouped into four classes: granule-bound starch synthase I (GBSSI), SSI, SSII and SSIII (Cao et al., 1999; Li et al., 1999b).

Genetic and biochemical analyses suggest that individual SS isoforms have unique roles in starch synthesis. Mutations in the *waxy* locus abolish GBSSI activity, and coincidentally result in a dramatically reduced amount of, or lack of, amylose in starch granules, indicating that GBSSI is responsible for amylose synthesis (for review, see Preiss and Sivak, 1998). SSI, SSII and SSIII are presumably involved in amylopectin synthesis in conjunction with starch branching enzyme and debranching enzyme (Myers et al., 2000). For example, mutations in the pea *rug5* locus eliminate SSII, and result in amylopectin that is enriched in short chains with a degree of polymerization (dp) of 7 – 9 and very long chains (> dp 1000), but is deficient in chains of intermediate length (dp 15 - 45) (Craig et al., 1998). In *sta3* mutants of *C. reinhardtii*, the activity of a 115 kDa

soluble SS is absent, and the amylopectin has a higher number of short chains (dp 2-7) and fewer chains of intermediate length, than that in the wild-type (Fontaine et al., 1993). The *dul* mutants produce a more highly-branched amylopectin and accumulate 15% of starch as intermediate material (Wang et al., 1993). These studies suggest that different SS isoforms make specific contributions to amylopectin synthesis.

In wheat endosperm, four SS isoforms have been reported, including GBSSI (Nakamura et al., 1995), SSI (Li et al., 1999a), SSII (Li et al., 1999b; Gao and Chibbar, 2000) and SSIII (Li et al., 1999a). Following the nomenclature suggested by Knight et al. (1998), the four SS isoforms of wheat are referred to as wGBSSI, wSSI, wSSII and wSSIII, respectively. As in other plant species, wGBSSI is exclusively starch granule-bound, and it is responsible for amylose synthesis (Nakamura et al., 1995). wSSII is present both in endosperm soluble fractions and within starch granules at the early stage of wheat endosperm development, but becomes exclusively granule-bound at mid and late stages (Li et al., 1999b). The absence of wSSII in wheat endosperm was reported to result in the deformation of large starch granules and the production of a novel starch with increased iodine binding capacity (Yamamori, 1998). wSSI is localized both in starch granules and soluble fractions in wheat endosperm (Denyer et al., 1995; Li et al., 1999a). Yamamori and Endo (1996) separated wSSI into three isoforms through two-dimensional polyacrylamide gel electrophoresis (PAGE), and confirmed the three wSSI isoforms were encoded at loci on the short arms of chromosomes 7B, 7D and 7A. Recently, a cDNA clone, and a corresponding genomic DNA clone corresponding to 75kDa wSSI were isolated from wheat D genome (Li et al., 1999a). To date, wSSIII has

only been identified as a soluble SS activity in wheat endosperm by the native PAGE gel analysis (Li et al., 1999a).

In potato tubers, SSII and SSIII have been shown to account for approximately 10-15% and 80% of the total starch synthase activity, respectively (Edwards et al., 1995; Marshall et al., 1996). Antisense inhibition of potato SSII produces little effect on the structure of starch and the morphology of starch granules. In contrast, reduction of SSIII activity to very low levels by expression of antisense RNA causes a serious disruption of starch granule morphology and dramatic alterations of amylopectin structure (Edwards et al., 1999). In wheat endosperm, although multiple SS isoforms have been identified, their relative contributions to the soluble SS activity have not been determined. Denyer et al. (1995) analyzed wheat SS isoforms partially purified by anion exchange chromatography, and reported that the major soluble SS was an unidentified SS isoform. More recently, Li et al. (1999a) separated multiple wheat soluble SS isoforms by native PAGE, and proposed that wSSI was the major soluble SS.

In this study, we describe the isolation of two cDNA clones encoding wSSI in wheat endosperm. RNA gel blot analysis showed that the expression and localization of wSSI in wheat endosperm varies with developmental stage, which extends the previous observations that wSSI is present both in starch granules and soluble phase (Denyer et al., 1995; Li et al., 1999a).

5.3 Materials and Methods

5.3.1 Materials

Plants of hexaploid wheat (*Triticum aestivum* L.) cultivar CDC Teal were grown in a greenhouse with controlled environmental conditions (25 °C day / 20 °C night, with supplementary light provided 16 h per day). Liquid fertilizer (20 N: 20 P: 20 K) was applied weekly to plants. Leaves, stems, roots, ovaries, pollen and florets were collected prior to anthesis. Immature kernels were harvested at 5, 10, 15, 20 and 25 days-post-anthesis (DPA). All the samples were frozen immediately in liquid nitrogen, and stored at -80 °C until use.

5.3.2 Preparation of starch granule-bound proteins and endosperm soluble fractions

Starch granule-bound proteins (SGP) and endosperm soluble fractions were prepared from frozen wheat kernels, as described by Peng et al. (2000). Protein concentrations in endosperm soluble fractions were determined using a dye binding assay kit from Bio-Rad.

5.3.3 Preparation of wSSI antibodies

The wSSI for producing antibodies was isolated from wheat starch granules. The SGP extracted from starch granules isolated from mature kernels of wheat (cv CDC Teal) were resolved on SDS-PAGE as described (Peng et al., 2000), and visualized by Coomassie blue staining. The SDS-PAGE gel strips with wSSI identified based on its migration on SDS-PAGE (Denyer et al., 1995; Li et al., 1999a) were excised, lyophilized, ground into powder, injected into a cereal-starved rabbit and anti-serum was prepared as described (Båga et al., 2000). The homogeneity of the isolated wSSI was verified by SDS-PAGE analysis.

5.3.4 Analytical procedures

Immunoblot analysis

Both SGP and endosperm soluble fractions were subjected to SDS-PAGE and immunoblot analysis, using the method of Peng et al. (2000). All the immunoblots were incubated with the wSSI antibody at a dilution of 1:8000 for 3 h and the immunoreactive bands were identified by chemical staining with 5-bromo-4-chloro-3-indolyl phosphate and 4-nitro blue tetrazolium (Stratagene) following the supplier's instructions.

Immunoprecipitation

Endosperm soluble fractions (100 µg proteins) were incubated on ice with 0.5 to 4 µL wSSI antiserum or pre-immune serum at a final volume of 50 µL for 1 h with gentle shaking. Fifty microliters of 10% (w/v) protein A-Sepharose CL-4B (Sigma) was added, and then the samples were incubated on ice for 1 h with gentle shaking, followed by centrifugation at 10,000 x g for 10 min. The supernatant (2.5 µL) was assayed for starch synthase activity (see below), or subjected to immunoblot analysis. The pellet was washed three times in homogenization buffer (50 mM Tris-HCl, pH 7.5, 10 mM EDTA, 5 mM DTT, 10% glycerol, 0.1% (w/v) polyvinyl pyrrolidone), prior to immunoblot analysis of the precipitated proteins.

Soluble starch synthase assay

Starch synthase activity was assayed essentially as described by Pollock and Preiss (1980). Assay mixtures (100 µl) contained 100 mM Bicine/NaOH, pH 8.0, 5 mM EDTA, 0.5 M sodium citrate, 0.5 mg mL⁻¹ BSA, 10 mg., mL⁻¹ glycogen, 0.7 mM ADP-[¹⁴C]Glc (500 cpm/nmol), and varying amounts of endosperm soluble fractions. Reactions were initiated by the addition of the radioactive ADP-Glc, incubated at 30°C

for 30 min, and terminated by spotting the aliquots onto GF/A glass fiber filters (Fisher), which were dried under a heat lamp. The filters were washed once in cold 66% ethanol containing 0.85 mM EDTA, once in 66% ethanol, and finally once in 70% ethanol. Preliminary experiments demonstrated that the amount of ^{14}C -glucose incorporation into ethanol-insoluble product was linear with the amount of cellular proteins used in the assay.

Zymogram analysis

Zymogram analysis was carried out according to Buléon et al. (1997), with some modifications. SGP and endosperm soluble fractions were resolved by SDS-PAGE (10%, 30:0.135 acrylamide/bisacrylamide), containing 0.3% rabbit muscle glycogen (Sigma). The gel was washed four times for 30 min each in 0.04 M Tris, and then incubated in reaction buffer (50 mM Tris-HCl, pH 9.0, 100 mM $(\text{NH}_4)_2\text{SO}_4$, 15 mM β -mercaptoethanol, 5 mM MgCl_2 , 0.5 mg/mL BSA and 4 mM ADP-Glc) for 36 h at room temperature. Subsequently, the gel was washed in water twice for 30 min. and stained with an iodine solution (0.25% KI and 0.025% I_2).

5.3.5 Isolation and expression of *wSsI* cDNA clones

PCR amplification of a cDNA fragment of wheat SsI

To amplify a cDNA fragment of wheat *SsI* (*wSsI*), degenerate oligonucleotide primers were derived from the most highly conserved regions in maize *SsI* (Genebank accession number: AF036891) and rice *SsI* (Genebank accession number: D16202): *SsI* / forward, 5'-GGGAGGATATATT/CTATGGA-3'; *SsI* / reverse, 5'-CAAGGTTCTGAATCTGGATGGCA-3'. PCR mixtures with two μl aliquot of a wheat cDNA library (2×10^7 Pfu/ μl , see below) were assembled in a 50 μl reaction mixture

containing 100 mM Tris-HCl, pH 9.2, 15 mM MgCl₂, 250 mM KCl, 200 μM dNTP and 10 pmol of the two oligonucleotide primers. The reaction was initiated with a denaturing step at 96 °C for 15 min, followed by one cycle of 94°C for 4 min (with one unit Taq DNA polymerase added at the end); 40 cycles of 55°C for 1 min, 72°C for 2 min, and 94°C for 1 min; one cycle of 55°C for 4 min and 72°C for 7 min. A 816-bp fragment was amplified, and designated as *wSs/p*.

Library screening , DNA sequencing and analysis

The wheat cDNA library used in this study was constructed in the vector λ-ZAP, using mRNA isolated from 12 DPA wheat (*Triticum aestivum* cv. Fielder) kernels (Nair et al., 1997). The PCR amplified DNA fragment, *wSs/p*, was labeled with [³²P]dCTP by random-priming, and used as probe to screen the wheat cDNA library. All phage lift membranes were hybridized and washed under high stringency conditions, as described by Church and Gilbert (1984). DNA from positive plaques was sequenced as described by Nair et al. (1997). The nucleotide sequences and deduced amino acid sequences were analyzed using the Lasergene biocomputer software (DNASTAR).

Construction of wSsI expression vectors

The SSI prokaryotic expression vectors were constructed in two steps. First, the cDNA encoding the mature wSSI was isolated from wSSI-pBluescript SK plasmid with restriction enzymes *PspOM* I and *Xho* I at the 5' and 3'-termini, respectively. *PspOM* I and *Not* I produce compatible ends thus, the isolated cDNA was subcloned to the *Not* I/*Xho* I sites of the expression vector pET-28(a) (Novagen) to give the construct pPM1. The gene construct, pPM1, was amplified in BL21(DE3) cells (Novagen).

For the second expression vector, a gene encoding mature wSSI was excised from pPM1 with restriction enzymes *EcoR* I at the 5'-terminus and partially digested with limited amounts of *Pst* I to produce DNA fragments with two variants at the 3'-terminus. The DNA fragment with the complete cDNA was subcloned into the *EcoR* I/*Pst* I sites of the expression vector pKK388-1 (CLONTECH) to give the wSSI expression vector designated as pPM2. The partial *Pst* I digestion also produced a truncated *wSsl* cDNA, which encodes a product with 120 amino acids deleted from the C-terminus of wSSI. The truncated *wSsl* cDNA was subcloned into the *EcoR* I/*Pst* I sites of the expression vector pKK388-1, to give the *wSsl* expression vector pPM3.

Expression of wSsl in E. coli

Expression vectors pPM2 and pPM3 were transformed into *E. coli*, RH98, a glycogen synthase (*glgA*)-deficient mutant. An overnight culture of RH98/pPM2 and RH98/pPM3 cells was diluted 1:20 (v/v) in fresh LB containing 100 µg/ml ampicillin. The cells were grown at 37°C with shaking to an A_{600} of 0.6, followed by induction of *wSsl* expression by the addition of isopropyl-D-thiogalactoside (IPTG) to a final concentration of 1 mM. Following growth at 25°C for 5 h, the cells were pelleted, suspended in lysis buffer (50 mM Tris-acetate, pH 7.5, 10 mM EDTA and 5 mM DTT), and lysed by sonication. The homogenate was centrifuged at 10,000 g for 10 min. The supernatant was used for SS activity assay and immunoblot analysis (see Analytical Procedure).

Complementation of E. coli mutant RH98

To test for complementation of glycogen synthase deficiency in RH98 by wSSI, RH98/pPM2, RH98/pPM3, RH98/pKK388-1 and wild type *E. coli* strain JM101 were

grown on an enriched medium [0.85% (w/v) KH_2PO_4 , 1.1% (w/v) KH_2PO_4 , pH 7.0, 0.6% (w/v) yeast extract, 1% (w/v) glucose, 100 $\mu\text{g/ml}$ ampicillin, and 1.5% (w/v) agar], and grown under inductive conditions (0.1 M IPTG). Following growth at 37°C for 20 h, cells were stained with an iodine solution (0.03 M I_2 , 0.04 M KI).

5.3.6 RNA extraction and expression analysis of *wSsI* in wheat

Total RNA was extracted from frozen wheat tissues using a phenol/chloroform method, as described by Wilkins and Smart (1996), with some modifications. Wheat tissues (1-3 g), including leaves, stems, roots, florets prior to anthesis, ovaries, pollen and immature kernels were ground in liquid nitrogen, and suspended in 15 ml extraction buffer (50 mM Tris-HCl, pH 9.0, 150 mM LiCl, 5 mM EDTA, 100 mM β -mercaptoethanol, 5% SDS). The slurry was extracted in 15 ml phenol/chloroform (1:1, v/v) for 5 min, and centrifuged at 2000 x g for 15 min. The aqueous phase was re-extracted twice with phenol/chloroform. The RNA and DNA mixture was precipitated with 0.1 volume 3 M sodium acetate (pH 5.2) and 1 volume isopropanol at -20°C for 2 h. Following centrifugation at 13,000 x g at 4°C for 30 min, the pellet was dissolved in 5 ml DEPC-treated water and 5 ml 4 M LiCl, then kept at 4°C for 1 h. The RNA was pelleted by centrifugation at 13,000 x g for 30 min, rinsed twice in 70% ethanol, air-dried and dissolved in DEPC-treated water. Total RNA (10 μg) was denatured, and resolved by 1.2% (w/v) agarose-formaldehyde electrophoresis, transferred to a Hybond N^+ membrane (Amersham), and hybridized with radioactive-labeled *wSs/p*, as described (Gao et al., 1996).

5.4 Results

5.4.1 Isolation of *wSsI* cDNA clones

To obtain a *wSsI* cDNA fragment for screening the wheat cDNA library, a pair of oligonucleotide primers was designed based on the nucleotide sequences conserved between maize and rice *SsI* cDNA. A single 817-bp cDNA fragment was amplified from a wheat cDNA library by PCR. This PCR-amplified cDNA fragment, *wSsI/p*, showed 87% and 87.5% sequence similarity to the corresponding region in maize and rice *SsI* cDNA, respectively.

Screening of a wheat cDNA library (6.0×10^5 pfu) resulted in the isolation of six clones which were characterized by restriction digestion analysis and DNA sequencing. Based on the nucleotide sequence similarity the six clones could be divided into two groups of three clones each. The longest cDNA clone (2575 bp) from group 1, referred as *wSsI-I*, contained an open reading frame of 1943 bp, which was flanked by 5' and 3' untranslated regions of 222 and 409 nucleotides, respectively. A short sequence (AATAAG) closely similar to the consensus higher plant polyadenylation signal was present at nucleotide 2285. The open reading frame encoded a 647-amino acid polypeptide starting at nucleotide 223 and ending at nucleotide 2166. The longest cDNA clone (2421 bp) from group 2, designated as *wSsI-II*, had an open reading frame of 1943 bp (from nucleotide 127 to 2070) flanked by a 5' untranslated sequence of 126 bp and a 3'-region of 351 bp. The putative polyadenylation signal (AATAAG) was located at nucleotide 2290. The open reading frame predicted an 647-amino acid polypeptide, *wSSI-II*. Both *wSSI-I* and *wSSI-II* had a calculated molecular mass was 71 kDa. The polypeptide, *wSSI-I*, is identical to the *wSSI* encoded by wheat D genome (Genebank

Figure 5.1. Comparison of deduced amino acid sequences of wSSI from *wSsI-I* (Li et al., 1999a; this paper) and *wSsI-II* cDNA (this paper) with maize SSI (Knight et al., 1998) and rice SSI (Baba et al., 1993). Transit peptide sequences are highlighted. Identical amino acids among these sequences are indicated with an asterisk. Dashes (-) represent gaps added to optimize sequence alignment.

Wheat SSI-I	GRYVAELSREGPAARP	57
Wheat SSI-II	57
Maize SSIP	52
Rice SSID*GS*HG	47
Wheat SSI-I	--AQQQLAPPLVPGFLAPP--PPAPAQSPAP--TQPPLPDAGVGELAPDLLLEGIAEDS	111
Wheat SSI-II	111
Maize SSI	PLPPA-L*.....AE*TG----E*AS*P*V*...L*D*GLE--P*...G*	104
Rice SSI	PL*PAPLVKQ*VL*T*V*TST*...T*...AP*P*...S*...IE*...LT*...	105
Wheat SSI-I	IDSIIVAASEQDSEIMDANEQPOAKVTRSIVFVTGEAAPYAKSGGLGDVCGSLPIALAAR	171
Wheat SSI-IIKD.....	171
Maize SSI	*NTV*V*.....VVG*E*AR*...Q*.....S*.....V*....	164
Rice SSI	*KT*FV*...E*...V*E*A*.....V*.....S*.....L*	165
Wheat SSI-I	GHRVMVMPRYLNGSSDKNYAKALYTGKHIKIPCFGGSHEVTFFHEYRONVDWVFDHPS	231
Wheat SSI-IIA*.....	231
Maize SSIT*...N*F*E*...R*...E*...S*.....	224
Rice SSIM*ALN*F*N*F*E*...E*...S*.....	225
Wheat SSI-I	YHRPGSLYGDNFGAFGDNQFRYTLCLYAAACEAPLILELGGYIYGQNCMFVNDWHASLVP	291
Wheat SSI-II	291
Maize SSIN*...K*.....	284
Rice SSIN*.....K*.....	285
Wheat SSI-I	VLLAAKYRPGVYRDSRSTLVIHNLHQGVPEASTYPDLGLPPEWYGALEWVFPWARRH	351
Wheat SSI-II	351
Maize SSIK*...I*.....	344
Rice SSIA*V*.....	345
Wheat SSI-I	ALDKGEAVNFLKGAVVTADRIVTVSQGYSWEVTTAEGGQGLNELLSSRKSVLNGIVNGID	411
Wheat SSI-II	411
Maize SSIK*.....	404
Rice SSI	405
Wheat SSI-I	INDWNPTTDKCLPHHYSVDDLSGKAKCKAELQKELGLPVREDVPLIGFIGRLDYQKGIDL	471
Wheat SSI-II	471
Maize SSIA*...I*C*.....GA*...I*P*.....	464
Rice SSIS*...F*Y*.....I*P*.....	465
Wheat SSI-I	IKMAIPELMREDVQFVMLGSGDPIFEGWMRSTESSYKDKFRGWVGFSPVPSHRITAGCDI	531
Wheat SSI-II	531
Maize SSI	*QLI*D*.....EL*D*...IF*.....	524
Rice SSI	**L*D*...DNI*...G*...G*R*.....	525
Wheat SSI-I	LLMPSRFPCGLNQLYAMQYGTVPVVHGTGGLRDTVETFPNPFAGKEEGTGWAFSPLTVD	591
Wheat SSI-II	591
Maize SSIA*...N*...EN*Q*...A*...TE	584
Rice SSIN*...AE*...Q*...IE	585
Wheat SSI-I	KMLWALRTAMSTFREHKPSWEGLMKRGMTKDHTWDHAAEQYEQIFEWAFVDQPYVM-	647
Wheat SSI-II	647
Maize SSI	N*F--VDI*NCNIYIQGT--QV*LG*ANEAR*V-----KRLH*GPCR---	622
Rice SSI	*NA--VGI*DGN*DIQGT--QV*LGGSNEAR*V-----KRLYMGPCRLTV	626

accession number: AFO98103) (Li et al., 1999a) except one amino acid difference at position 321, where leucine is replaced by valine. However, at this position, valine is present in wSSI-II (see below), maize and rice SSI (Figure 5.1).

wSSI-I and wSSI-II shared 99.2% identity, with variation at four positions, amino acids 39, 130, 131 and 198 in their deduced amino acid sequences (Figure 5.1). At the nucleotide level, *wSsI-I* and *wSsI-II* have 98.1% sequence similarity, and there is only a two-nucleotide difference between *wSsI-I* and *wSsI* reported by Li et al. (1999a) (data not shown). Comparison of amino acid sequences from maize SSI (Genebank accession number: AF036891), rice SSI (Genebank accession number: D16202), wSSI-I and wSSI-II showed that there was very high similarity among the four SSI amino acid sequences, ranging from 75.9% to 79.7% (Figure 5.1).

Because wSSI is localized to amyloplast, therefore, a transit peptide is expected to be present in wSSI pre-protein. Comparing the N-terminal sequence of wSSI reported by Rahman et al. (1995) with the deduced amino acid sequences of wSSI-I and wSSI-II showed that the first 41 amino acids in wSSI-I and wSSI-II sequences composed their transit peptides, which shared 30.8% and 27.2% amino acid sequence similarity with those of maize SSI and rice SSI. Further, the position of transit peptide cleavage sites in wSSI-I and wSSI-II is similar to that in maize SSI (Knight et al., 1998) and rice SSI (Baba et al., 1993) (Figure 5.1). The wSSI transit peptide cleavage site (V/L-A-R↓G) does not completely match with the consensus cleavage site (V/L-X-A/C↓A) for chloroplast transit peptides (Gavel and von Heijne, 1990). However, the wSSI transit peptide consists of very few acidic, but many hydroxylated amino acids being rich in arginine through amino acid -1 to -10 at the transit peptide cleavage site, characteristic

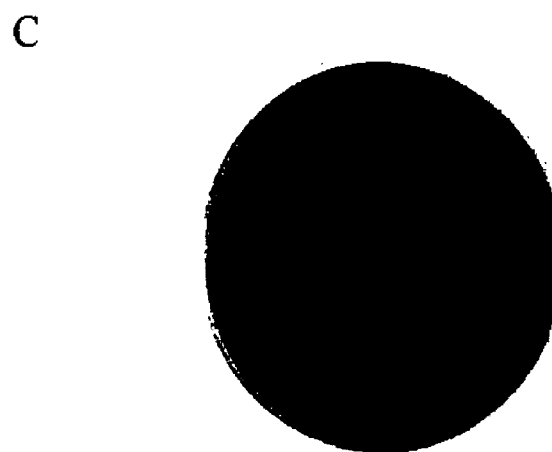
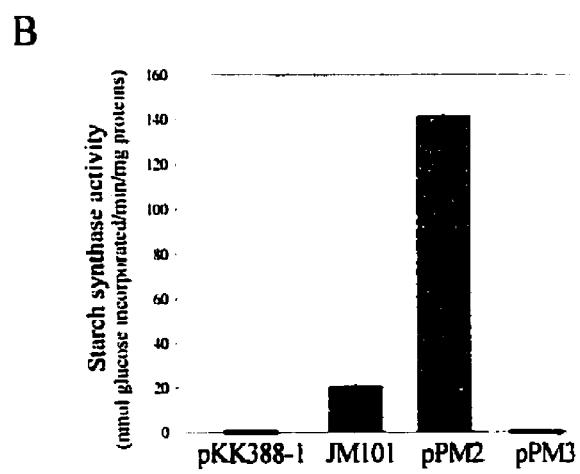
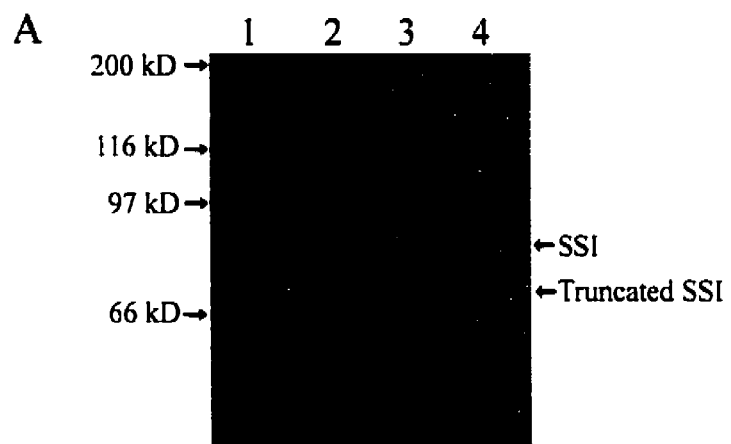
features of a transit peptide, and does have valine at amino acid -3 of the cleavage site (Gavel and von Heijne, 1990). Both mature wSSI-I and wSSI-II had a calculated molecular mass of 67 kDa.

5.4.2 wSsI cDNA encodes an active starch synthase

To verify that the isolated wSsI cDNA clone encodes an active SS, expression vectors containing wSsI cDNA and a variant with C-terminal deletion were constructed, and introduced separately into a glycogen synthase deficient mutant of *E.coli*, RH98. Immunoblot analysis (Figure 5.2A) showed that expression of wSsI cDNA in RH98 produced a protein, with a molecular mass of 80 kDa, but it was recognized by the wSSI antibody produced in rabbits immunized with the 75 kDa wSSI from starch granules. The C-terminal truncated wSsI cDNA produced a polypeptide with a molecular mass of 70 kDa, which was also recognized by wSSI antibody. As expected, the wSSI antibody did not recognize any protein in the RH98/pKK388-1 lysate.

The bacterial cell extracts harboring the vector with no cDNA (RH98/pKK388-1) or the truncated wSsI cDNA (RH98/pPM3) resulted in no detectable SS activity (Figure 5.2B). The expression of wSsI cDNA in RH98 (RH98/pPM2) resulted in the production of seven-fold higher SS activity as compared to an *E.coli* strain JM101, which is wild-type for SS activity. These observations were further confirmed by iodine staining of glycogen (Figure 5.2C). *E.coli* JM101 and RH98/pPM2 possess glycogen and starch synthase activity, respectively, stained red when treated with iodine. In contrast, RH98/pKK388-1 and RH98/pPM3, which did not exhibit any starch synthase activity, remained colorless when stained with iodine, because the two strains did not synthesize glycogen (Figure 5.2C).

Figure 5.2. Expression of *wSsl* cDNA in *E.coli* strain RH98. **A.** Immunoblot analysis of recombinant wSSI produced in *E.coli*. Total cell extracts (50 µg proteins) harboring the indicated plasmids were analyzed by SDS-PAGE. Lane 1, RH98/pKK388-1; Lane 2, RH98/pPM3; Lane3, RH98/pPM2; Lane 4, proteins from wheat starch granules (0.5 mg). The immunoblot analysis was done with antibodies against wheat SSI. Migration of marker proteins is indicated on the left-hand side. **B.** Aanlysis of starch synthase (SS) activity in *E.coli* cell extracts. Total cell extracts (2.5 µg proteins) from IPTG-induced *E.coli* cells of the indicated genotypes were assayed for SS activity (Pollock and Preiss, 1980). Values represent the average enzyme activity \pm SD, were determined from four independent replications. **C.** Complementation of the glycogen synthase activity in *E.coli* strain RH98 I. Glycogen synthase producing strain JM101/pBluscript SK; II. RH98/pPM2; III. RH98/pPM3; IV. RH98/pBluscript SK.



5.4.3 Contribution of wSSI to the total soluble starch synthase activity in wheat endosperm

Endosperm soluble fractions prepared from 13-17 DPA kernels were used in immuno-precipitation experiments to assess the contribution of soluble wSSI to the total soluble SS activity in wheat endosperm. The antibody-protein complex was precipitated from the endosperm soluble fractions incubated with preimmune or anti-wSSI serum. Incubation with pre-immune serum did not affect the SS activity in the supernatants after immuno-precipitation, whereas wSSI antibody reduced starch synthase activity significantly (Figure 5.3A). The maximum inhibition of SS activity was approximately 65% when 3 μ l or more wSSI immune serum was added to the immuno-precipitation reaction mixture. Immunoblot analysis of immuno-precipitates and supernatants showed that the antibody dosage-dependent removal of wSSI from endosperm soluble fractions (Figure 5.3B) correlated closely with the inhibition of SS activity in immuno-precipitation mixture supernatants. The wSSI antibody did not recognize any other polypeptides in the immuno-precipitates or the supernatant of the immuno-precipitation reaction mixtures (Figure 5.3B).

5.4.4 RNA gel blot analysis of *wSsI* in wheat

The expression of *wSsI* in various tissues of wheat cv. CDC Teal was investigated by RNA gel blot analysis using *wSsI* as the probe. As shown in Figure 5.4A, a single transcript of about 2.6 kb was detected in endosperm, leaf, stem, root, pre-anthesis floret, pollen and ovary. A single transcript of about 2.6 kb was also observed throughout endosperm development, and the accumulation of *wSsI* transcripts was higher at five-10 DPA than at 15-25 DPA (Figure 5.4B). These results are in contrast to

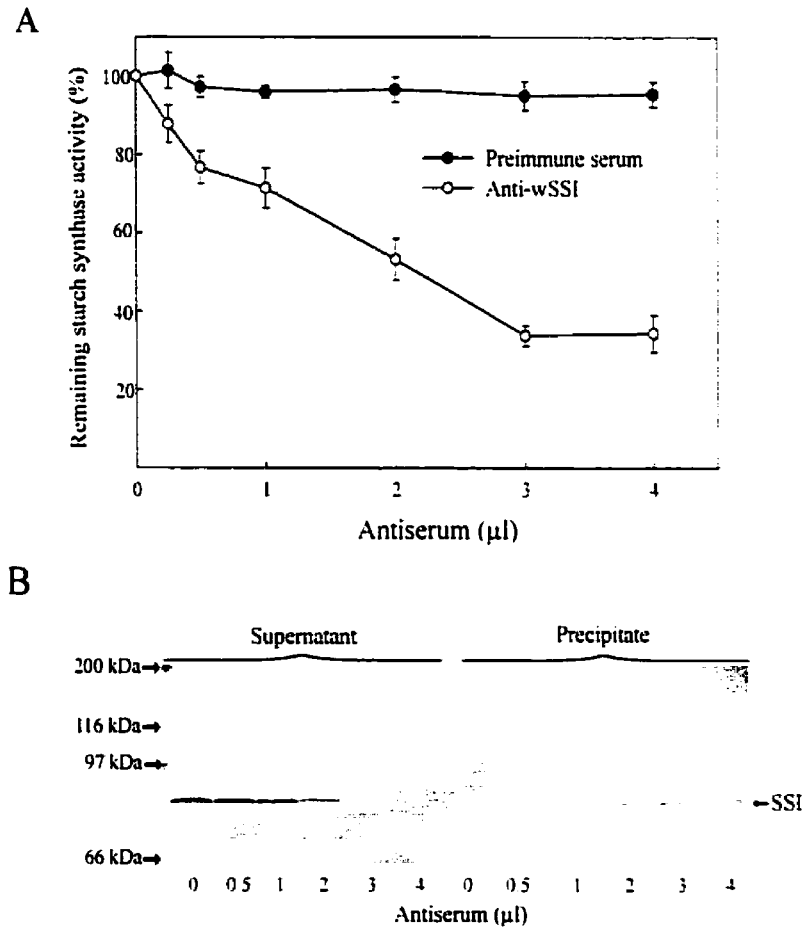


Figure 5.3. Immunoprecipitation of wSSI activity from wheat endosperm soluble fraction with wSSI antibody. **A.** Assay for remaining SS activity in the immunoprecipitation mixture supernatant. Remaining SS activity was expressed as a percentage of that after the incubation of endosperm soluble fraction with preimmune serum. Values represent the means \pm SD of three replications. **B.** Immunological detection of wSSI in the supernatant and immune complexes.

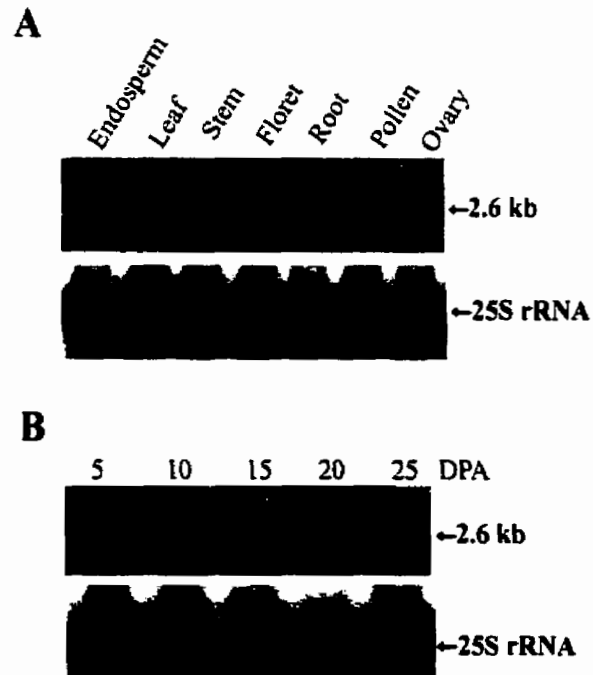


Figure 5.4. Detection of *wSsI* transcript in wheat by northern blotting. **A.** Expression of *wSsI* in different tissues, including endosperm (15 DPA), leaf, stem, pre-anthesis floret, root, pollen and ovary. Ten μ g of total cellular RNA was loaded per lane. **B.** Expression of *wSsI* in developing wheat kernels. Each lane was loaded with ten μ g of total cellular RNA.

an earlier report in which *wSsl* transcripts were detected only in the endosperm, and the *wSsl* transcripts accumulated at higher level from eight- 18 DPA, then decreased with kernel development (Li et al., 1999a).

5.4.5 Localization of wSSI in wheat endosperm

To determine the sub-cellular localization of wSSI in wheat endosperm, soluble fractions and SGP were extracted from bulked wheat endosperm and subjected to SDS-PAGE separation and immunoblot analysis (Figure 5.5A). In starch granules, wSSI was present at almost similar levels throughout endosperm development, whereas in endosperm soluble fractions, it was detected only during 10 to 25 DPA. This result is consistent with previous reports that wSSI is localized both in endosperm soluble fractions and starch granules (Rahman et al., 1995; Denyer et al., 1995; Li et al., 1999a). However, in this study, we found that the presence of wSSI in endosperm soluble fractions varied with the endosperm development stage. At five DPA, the endosperm soluble fractions, wSSI was not detected, but at 10 and 15 DPA, very high concentrations of wSSI was observed by immunoblot analysis. After 20 DPA, the concentrations of wSSI decreased with the kernel development (Figure 5.5A).

Zymogram analysis was done on SDS-PAGE separated proteins, which were subsequently renatured in gels, to allow for detection of enzyme activities. Endosperm soluble fractions and SGP extracted from single developing wheat kernels were subjected to zymogram analysis. A considerably higher wSSI activity was detected in starch granules than in endosperm soluble fractions (Figure 5.5B). With the development of wheat endosperm, the yield of starch granules per kernel increased (Peng et al., 2000), and this uneven distribution of wSSI in the two phases became more pronounced. In

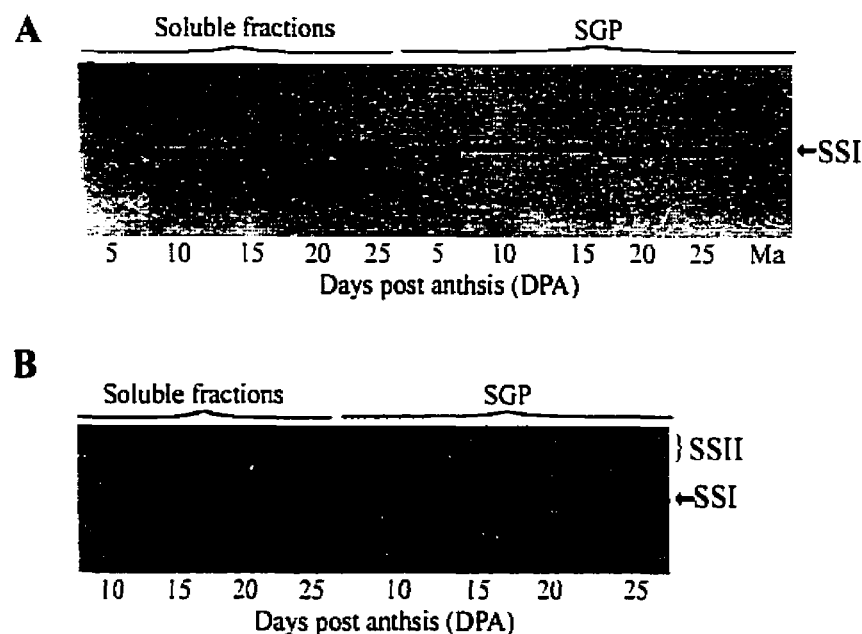


Figure 5. 5. Analysis of the localization and expression of wSSI in developing wheat endosperm. **A.** Immunological detection of wSSI. Each lane was loaded with 50 μ g proteins from endosperm soluble fractions or the protein extracted from 0.5 mg starch granules. **B.** Zymogram analysis of SS activity in single wheat kernels. Each lane in Soluble fractions contained all soluble proteins from a single kernel. Lanes in SGP were loaded with SGP from $\frac{1}{2}$, $\frac{1}{2}$, $\frac{1}{4}$ and $\frac{1}{4}$ of a single kernel of 10, 15, 20 and 25 DPA.

endosperm soluble fractions, wSSI concentration was low at 10 DPA, and then increased and stayed at an almost constant level from 15 to 25 DPA (Figure 5.5B).

5.5 Discussion

In this study, we isolated two full-length cDNA clones, *wSsI-I* and *wSsI-II*, and demonstrated that they encode active SS when expressed in *E.coli*. The high level of amino acid sequence similarity (>80%) between wSSI, maize SSI and rice SSI (Figure 5.1), suggest that SSI in different cereals could be encoded by homologous genes. However, the genes encoding SSI in wheat, rice and maize show differences in tissue-specific and temporal expression (Knight et al., 1998; Baba et al., 1993; Li et al., 1999a). Maize *SsI* transcripts are specifically accumulated in the endosperm from 15 to 35 DPA (Knight et al., 1998), whereas in rice, *SsI* transcripts are observed in seeds from five to 20 DPA and also in leaves (Baba et al., 1993). In wheat, Li et al. (1999a) showed the expression of *SsI* only in endosperm, but could not detect it in leaves, pre-anthesis florets and endosperms from field grown wheat (cv. Rosella) plants. They also reported that in wheat (cv Gabo), which was grown under controlled environmental conditions, the *wSsI* transcript accumulation in endosperms peaked between eight to 18 DPA, then decreased as the kernel matured. In our study, of wheat cv. CDC Teal grown in a greenhouse under defined environmental conditions, *wSsI* transcripts were detected in endosperm, leaf, stem, root, pre-anthesis floret, ovary and pollen (Figure 5.4A). In the developing endosperm, the *wSsI* transcript accumulation at five to 10 DPA was higher than that at 15 to 25 DPA (Figure 5.4B). The difference in *wSsI* transcript accumulation between our

result and that of Li et al. (1999a) suggests that the genetic background and/or environmental conditions could affect the *wSsI* expression.

Despite the minor differences in the *SsI* expression pattern, there are considerable similarities in the structure, behavior and function of SSI isolated from different plants. Imparl-Radosevich et al. (1998) showed that the deletion of 36 - 93 N-terminal amino acid residues of maize SSI did not affect its enzymatic activity, but only altered some of its kinetic characteristics. This suggested that the catalytic domain of maize SSI is not located at its N-terminus. In our study, we show that the removal of 120 amino acid long peptide at the C-terminus of wSSI abolished the SS activity. The expression of the C-terminal truncated *wSsI* in *E.coli* resulted in the production of a 70 kDa polypeptide recognized by wSSI antibody (Figure 5.2A), but it did not have SS activity (Figure 5.2B). Consequently, it also did not restore glycogen synthesis in the glycogen synthase deficient *E.coli* RH98 (Figure 5.2C). This result is consistent with the previous reports (Gao et al., 1998; Harn et al., 1998) that the C-terminus of maize SS is important for starch synthase activity.

The molecular mass of the mature wSSI predicted from the amino acid sequences of *wSsI*-I and *wSsI*-II is 67 kDa. While the apparent molecular mass estimated from SDS-PAGE of the starch granule-bound wSSI, soluble wSSI (Figure 5.3A) and the expressed wSSI from *E.coli* (Figure 5.2A) is 80 kDa. Similar aberrant migrations of starch synthase polypeptides in SDS-PAGE have also been observed in potato SSII (Edwards et al., 1995), maize SSI (Knight et al., 1998), maize SSII (Imparl-Radosevich et al., 1999) and wSSII (Li et al., 1999b, Gao and Chibbar, 2000). The difference in the predicted molecular mass and apparent molecular mass as determined by SDS-PAGE for

the starch synthases is most likely due to the inherent polypeptide structure of starch synthases as suggested before (Gao and Chibbar, 2000). The slower migration on SDS-PAGE gels could be due to the SS interactions SS with glucan polymers and/or post-translational modifications of SS, as previously suggested (Edwards et al., 1995; Li et al., 1999b). However, our observations that recombinant SSI (this work) and SSII (Gao and Chibbar, 2000) produced in *E.coli* show the same aberrant migration as SSI extracted from starch granules makes the latter explanations less likely.

In plant storage organs, multiple isoforms of SS are found, which likely make differential contributions to the total soluble SS activity. In maize, SSI accounts for approximately 60% of total soluble SS activity, and DUI contributes 20-30% (Cao et al., 1999). SSII and SSIII are the major soluble SS in pea embryos and potato tubers, respectively (Denyer and Smith, 1992; Marshall et al., 1996). In this study, we found that wSSI contributed about two-thirds of total soluble SS activity (Figure 5.3). This is consistent with the previous report (Li et al., 1999a) that wSSI is the major soluble SS in wheat endosperm.

Wheat, maize, pea and potato have the wSSI, maize SSI, pea SSII and potato SSIII as their major soluble SS, although these are different SS isoforms, but they are present both in starch granules and in soluble fractions of amyloplast. Besides their localization to amyloplasts, pea SSII and potato SSIII seem to have comparable function in the biosynthesis starch granules, because alterations in their activities result in related phenotypes: starch granules are similarly deformed, and amylopectin is enriched in short glucan chains (Craig et al., 1998; Edwards et al., 1999). Because wSSI is immunologically related to pea SSII (Denyer et al., 1995), and acts as the major soluble

SS in wheat endosperm, it can be speculated that wSSI may have a similar function in amylopectin synthesis as pea SSII and potato SSIII.

It is well documented that wSSI is present both in wheat endosperm soluble fraction and starch granules (Rahman et al., 1995; Denyer et al., 1995; Li et al., 1999a). This study extended our understanding of the localization and expression of wSSI throughout endosperm development. When bulked wheat endosperms were analyzed, wSSI was present at almost constant levels in starch granules from five DPA to kernel maturity. In endosperm soluble fractions, wSSI was not detected at five DPA, and had the highest concentration at 10 and 15 DPA (Figure 5.5A). However, the analysis based on single wheat kernel showed that significantly increasing amount of wSSI was trapped in starch granules with the growth of wheat endosperm, while in endosperm soluble fractions, wSSI concentration was present at an almost constant level from 15 to 25 DPA (Figure 5.5B).

The localization of wSSI in single wheat kernels may be associated with the development of starch granules. According to Badenhuizen (1965), an adequate number of starch molecules is needed before they crystallize to form small starch granules. It is possible that wSSI may be involved in the synthesis of these starch molecules, and subsequently it gets trapped in starch granules during crystallization. This could be the reason that no wSSI or low amount of wSSI is detected in endosperm fractions at the early stages (five–10 DPA) of endosperm development. Later, wSSI may function on the surface of starch granules (Ball et al., 1996), and is constantly entrapped into granules, which results in the detection of increased amount of wSSI in starch granules with the growth of wheat kernels. To maintain adequate quantities of wSSI to function on the

surface of starch granules, wSSI is synthesized constantly to compensate those embedded in starch granules, and therefore, the concentration of wSSI is kept at an almost constant levels from 15 to 25 DPA in endosperm soluble fractions. However, we do not know whether wSSI is active when embedded in starch granules. More studies are needed to precisely characterize the role of SSI in starch granule biosynthesis. The isolation and characterization of cDNA clones encoding SSI can be used in genetic engineering techniques to regulate SSI activity in developing wheat grains to study the function of SSI in starch granule biosynthesis and/or to produce wheat with altered starch structure (Båga et al., 1999).

CHAPTER 6

GENERAL DISCUSSION

Molecular and biochemical characterization of wheat A- and B-type starch granules may accelerate the breeding for wheat cultivars with predominant A- or B-type starch granules. Such wheat cultivars are very useful for food and non-food industries. This study focused on the molecular and biochemical characterization of wheat A- and B-type starch granules with two objectives: 1) to develop a method to purify A- and B-type starch granules from wheat endosperm; 2) to analyze genes and proteins that may be related to the biosynthesis of A- and B-type starch granules.

Purified wheat A- and B-type starch granules are essential for the precise characterization of the two starch granule types. Several methods, including microsieving, sedimentation in a water column, and elutriation have been developed to separate A- and B-type starch granules, which were subsequently analyzed for chemical composition and functional properties (for review, see Seib, 1994). However, these methods usually could not produce homogeneous A- and B-type starch granule populations (Eliasson and Karlsson, 1983). In this study, centrifugation through two Percoll solutions (70% and 100%) was found to be able to completely separate A- and B-type starch granules, which were confirmed to represent their counterparts in mature wheat endosperm (Table 3.1; Figure 3.1). Thus, the characteristics of intact A- and B-type starch granules in wheat endosperm could be determined by analysis of the chemical composition and gelatinization properties of the purified A- and B-type starch

granules. Consistent with previous reports (for review, see Seib, 1994), A-type starch granules contained higher amylose concentration than B-type starch granules, and both granules differed in gelatinization properties (Figure 3.1). Because the purity of the A- and B-type starch granule populations was increased in this study, the range of amylose concentration difference between the two starch granule types was larger than those published previously (Eliasson and Karlsson, 1983; Soulaka and Morrison, 1985).

Some starch granule-bound proteins (SGP) play important roles in the biosynthesis of starch granules in wheat endosperm, such as GBSSI (Nakamura et al., 1995) and SGP1, known as SSII (Yamamuri, 1998). Thus, the first step in this study was to determine whether some SGP are related to the biosynthesis of wheat A- and B-type starch granules. For this purpose, SGP must be extracted from purified A- and B-type starch granules. The reason for Sulaiman and Morrison (1990) and Rahman et al. (1995) to obtain similar SGP profiles from wheat A- and B-type granules may be that they used sedimentation in a water column to separate the two types of starch granules. In this study, SGP were extracted from the confirmed homogenous A- and B-type starch granule populations (Figure 3.1) and resolved by SDS-PAGE. Two SGP, SGP-140 and SGP-145 were observed to be preferentially associated with A-type starch granules in mature and developing wheat endosperm (Figure 4.1; Figure 4.3).

Immunoblotting and N-terminal sequencing suggested SGP-140 and SGP-145 were different variants of SBE1c, a 152 kD isoform of wheat starch branching enzymes (Båga et al., 2000) (Figure 4.4; Table 4.1). When expressed in *E.coli*, *Sbe1c* cDNA produced an active SBE, and complemented the SBE deficiency in the *E.coli* strain used by Båga et al. (2000). These results suggest that both SGP140 and SGP-145 have SBE

activity. Similar to granule-bound starch synthase I (GBSSI), SGP-140 and SGP-145 were localized in starch granules, but were not found in the endosperm soluble fractions (Figure 4.5). Therefore, SGP-140 and SGP-145 may be active in amylopectin synthesis on polymers of A-type starch granules, because wheat would not waste energy to synthesize non-functional SBE.

Mutants without amylose in many plant species have similar starch granules to wild-type, while no mutants selectively lacking amylopectin have ever been reported. This suggests that the major structure of starch granules is determined by amylopectin (Ball et al., 1998). In developing wheat endosperm, preferential association of SGP-140 and SGP-145 with small A-type, rather than B-type starch granules may result in that small-size A-type starch granules have more amylopectin than B-type, thus, small-size A-type starch granules could grow to become full-size A-type (Figure 4.2; Figure 4.3). Alternatively, the amylopectin produced by SGP-140 and SGP-145 may have specific structure, which may cause more amylopectin synthesized or deposited at the periphery of small-size A-type starch granules than at the periphery of B-type starch granules.

Currently, only endosperm of wheat, barley, rye and triticale contain A- and B-type starch granules, showing bimodal granule size-distribution (French, 1984). In other plant species, such as maize, rice, canary seed (endosperm) and potato (tubers), starch granules are not differentiated into A- and B-type, giving unimodal granule size-distribution. Examination of SGP profiles of barley, rye and triticale showed that homologs of SGP-140 and/or SGP-145 were preferentially associated with their A-type starch granules. In contrast, homologs of SGP-140 and SGP-145 were not observed in starch granules from potato tubers and maize, rice and canary seed endosperm (Figure

4.6). These results further suggest that SGP-140 and/or SGP-145 is correlated to the occurrence of the bimodal granule size-distribution through regulation of amylopectin synthesis in A-type starch granules.

The proportion of A- or B-type starch granules in wheat and barley endosperm is genetically controlled by multiple genes (Stoddard, 1998; Borém et al., 1999). Thus, it is necessary to investigate more starch synthetic enzymes to determine if they are involved in the biosynthesis of A- and/or B-type starch granules. In barley shrunken (*s/x*) mutants, the overall sizes of A-type starch granules are decreased, and granule size-distribution becomes unimodal (Schulman et al., 1994). Biochemical analysis reveals that the recessive mutation at the *s/x* locus causes the reduction of SSI activity by 86% with the disappearance of one SSI isoform (Tyynelä and Schulman, 1993). Additionally, the activity of enzymes upstream of SSI along the starch synthetic pathway, including sucrose synthase and ADP-Glu pyrophosphorylase are also considerably decreased by this mutation (Tyynelä et al., 1995). However, the products of these affected enzymes except SSI are accumulated, and the activities of GBSSI and SBE which are downstream of SSI in the starch synthetic pathway is not affected in *s/x* mutants. Therefore, SSI may be the mutation site in *s/x* mutants, and involved in the determination of A-type starch granule sizes in barley (Schulman and Ahokas, 1990; Tyynelä and Schulman, 1993). In wheat, the function of wSSI in starch synthesis is not known because natural mutants lacking wSSI activity from A, B and D genomes (wSSI full mutation) have not been identified and characterized. Since wheat and barley are related phylogenically, wSSI may affect the sizes of A-type starch granules, similar to barley SSI.

Biochemical and molecular characterization of A- and B-type starch granules in wheat endosperm makes it necessary to understand the function of wSSI. Analysis of wSSI full mutation is one of the best ways to achieve this goal. Although no naturally occurring wSSI full mutation has ever been found in wheat, it is possible to make one through genetic manipulation of *wSsl*. For this purpose, the first step is to clone *wSsl* cDNA and characterize wSSI. In this study, two nearly full-length cDNA, *wSsl*-I and *wSsl*-II were cloned from a wheat endosperm cDNA library (Figure 5.1). Expressed in the *E.coli* strain RH98, which possesses a glycogen synthase deficiency, *wSsl* cDNA produced an active starch synthase, and complemented the glycogen synthase deficiency (Figure 5.2). Northern blot analysis showed *wSsl* transcript was detected in leaf, stem, root, pre-anthesis floret, ovary and pollen and endosperm, and the expression level of *wSsl* at 5 – 10 DPA in endosperm was higher than at 15 – 25 DPA (Figure 5.4).

Denyer et al. (1995) and Li et al. (1999a) reported that wSSI was localized both in endosperm soluble fractions and starch granules. However, the localization and expression of wSSI throughout endosperm developmental stages are not known, nor is the relative distribution of wSSI between starch granules and endosperm soluble fractions. In this study, it was found that in developing wheat endosperm, wSSI was present at a constant level in starch granules from five to 25 DPA, while in endosperm soluble fractions, wSSI was not detected at five DPA, and had highest levels from 10 to 15 DPA. Analysis of SSI levels on single wheat kernel basis showed wSSI had similar concentrations in endosperm soluble fractions from 15 to 25 DPA, and much more wSSI was present in starch granules than in endosperm soluble fractions throughout endosperm development (Figure 5.5).

Multiple SS isoforms have identified in plant storage organs, and their relative contributions to the total soluble SS activity appear to be correlated with their importance in amylopectin synthesis (Edwards et al., 1999). In potato tubers, SSIII contributes approximately 80% of the total SS activity in potato tubers, much more than that of SSII, which accounts only 10-15% (Edwards et al., 1995; Marshall et al., 1996). Antisense inhibition of SSIII has much greater effect on amylopectin structure and starch granule morphology than antisense reduction of SSII activity (Edwards et al., 1995 and 1999). In wheat endosperm soluble fractions, three SS isoforms, wSSI, wSSII and wSSIII have been identified (Li et al., 1999a, 1999b), while which of these SS isoforms accounts for the major soluble SS activity has not been determined (Denyer et al., 1995a; Li et al., 1999a). From the immunoprecipitation experiment in this study, it is evident that wSSI is the major soluble SS in wheat endosperm as it contributed about 65% of the total soluble SS activity in wheat endosperm (Figure 5.3). Based on the effect of antisense inhibition of SSIII in potato tubers, it is expected that genetic manipulation of wSSI in wheat endosperm would have a potential to modify amylopectin synthesis significantly.

6.1 Future directions

The chemical composition and functional properties of wheat A- and B-type starch granules have been studied extensively (Kulp, 1973; Soulaka and Morrison, 1985; Tester and Morrison, 1990; Raeker et al., 1998). However, A- and B-type starch granules used in all previous studies were separated by microsieving, sedimentation in a water column, or elutriation, which were found not to be able to produce homogeneous

A- and B-type starch granule populations (Eliasson and Karlsson, 1983). The precise and accurate analysis of the chemical composition and functional properties of A- and B-type starch granules is essential for determining their end-uses in food and non-food industries (Seib, 1994). Thus it is necessary to develop methods to completely separate A- and B-type starch granules and characterize the two starch granule types.

In our study, purified A- and B-type starch granule populations were produced by centrifugation through two Percoll solutions, and they were confirmed to represent their counterparts in mature wheat endosperm (Table 3.1; Figure 3.1). In the future study, the purified A- and B-type starch granules will be used to evaluate their chemical composition and functional properties in details, including lipid and protein concentrations, retrogradation behavior, pasting characteristics and baking application. Furthermore, the structure of amylose and amylopectin of A- and B-type starch granules will be analyzed. In barley, Takeda et al. (1999) found that A- and B-type starch granules had different structure of amylose and amylopectin, and suggested that the biosynthesis of A- and B-type starch granules may be under different regulatory or genetic control. In wheat, the proportion of A- and B-type starch granules is genetically controlled (Stoddard, 1998), the two starch granule types may differ in the structure of their amylose and amylopectin.

The data in Chapter 4 suggest that preferential association of SGP-140 and SGP-145 with A-type starch granules may be correlated to the size growth of wheat A-type starch granules, and to the occurrence of the bimodal starch granule-size distribution in wheat, barley, rye and triticale. The question for future studies would be what roles SGP-140 and SGP-145 play in the size growth of wheat A-type starch granules.

As discussed above, in developing wheat endosperm, SGP-140 and SGP-145 may produce amylopectin with specific structure in small-size A-type starch granules, thus resulting in the growth of small-size A-type into full-size A-type starch granules. The cloning of cDNA of *Sbelc* (Båga et al., 2000), *Sbel* (Repellin et al., 1997) and *Sbell* (Nair et al., 1997) from wheat endosperm makes it possible to test this hypothesis. With the method described by Båga et al. (2000), cDNAs of *Sbelc*, *Sbel* and *Sbell* were expressed in the *E.coli* mutant with a SBE deficiency, respectively. If the enzyme encoded by *Sbelc* has the ability to produce branching glucan polymers with a specific structure in A-type granules, *Sbelc* would produce a branching structure in glycogen molecules different from those by *Sbel* and *Sbell* when the three *Sbe* isoforms are expressed in *E.coli* separately. Thus, analysis and comparison of the branching pattern of the glycogen molecules produced by *Sbelc*, *Sbel* and *Sbell* would determine whether *Sbelc* could produce a specific branching pattern in glycogen, and indicate if *Sbelc* has the potential to produce amylopectin with a specific structure in A-type starch granules.

Analysis of mutants lacking SGP-140 and SGP-145 may be another way to elucidate the roles SGP-140 and SGP-145 play for the size growth of wheat A-type starch granules. However, in hexaploid wheat, it will be difficult to find natural mutants lacking SGP-140 and SGP-145 or generate mutants via mutagenesis. Barley is diploid, and most likely shares the same mechanism for the synthesis of A- and B-type starch granules as wheat because barley and wheat are very closely related phylogenetically. Therefore, it is reasonable to use barley for future studies on SGP-140 as barley has SGP-140. There are two possible ways to obtain barley mutants lacking SGP-140. The first approach would be to identify natural occurring mutants in barley. This approach is

possible because barley *s/x* mutant affecting the size of A-type starch granules was found in nature (Schulman and Ahokas, 1990). However, this approach would take many years. The second approach would be antisense inhibition of SGP-140 in barley. Barley cDNA encoding SGP-140 could be readily cloned using wheat *Sbelc* cDNA as probe (Båga et al., 2000). Subsequently, this cDNA can be introduced into barley and expressed in an antisense direction under the control of a seed specific promoter. Thus, the production of SGP-140 in barley endosperm may be inhibited. The antisense approach has been successfully used in potato to study the effect of GBSSI, SBE I, SSII and SSIII on starch synthesis (Visser et al., 1991; Flipse et al., 1996; Edwards et al., 1999; Lloyd et al., 1999). The major drawback for this approach would be the low transformation efficiency of barley.

Rye contains the homolog of wheat SGP-145. Similar to barley, rye is diploid, and most likely shares the same mechanism for the synthesis of A- and B-type starch granules as wheat. Thus, rye can be used for future studies on SGP-145 with the same approaches as barley.

An alternative approach for elucidating the function of SGP-140 or SGP-145 in the synthesis of A- and B-type starch granules is to express the cDNA encoding SGP-140 or SGP-145 in maize endosperm. Maize can be genetically transformed (Gordon-Kamm et al., 1999) and has no homologs of SGP-140 and SGP-145. Because maize endosperm has a unimodal starch granule-size distribution, and the presence of SGP-140 and/or SGP-145 is correlated to the occurrence of the bimodal starch granule-size distribution, the introduction and expression of the cDNA encoding SGP-140 or SGP-145 in maize endosperm may result in the alteration of starch granule size-distribution.

wSSI was shown in this study to account for approximately two thirds of the total soluble starch synthase activity in wheat endosperm (Figure 5.3). This indicates that wSSI may play an important role in starch synthesis, similar to pea SSII (Craig et al., 1998) and potato SSIII (Edwards et al., 1999; Lloyd et al., 1999). The molecular cloning and characterization of *wSsI* cDNA from wheat endosperm make it possible to investigate the function of wSSI in starch synthesis, especially in the synthesis of A- and B-type starch granules. Yamamori and Endo (1996) identified partial wSSI mutant lines that are deficient in wSSI activity from either the A or B genome. Through crossing, wheat lines lacking wSSI from both A and B genomes could be developed. In these wheat lines, introduction and expression of *wSsI* cDNA from D genome in an antisense orientation under the control of a seed specific promoter (Båga et al., 1999a) may result in the inhibition of the synthesis of wSSI from A, B and D genomes. Subsequent analysis of the full wSSI mutants may provide conclusive evidence for the role of wSSI in the synthesis of starch granules.

6.2 Conclusions

- Centrifugation through two Percoll concentrations completely separated A- and B-type starch granules, and the two types of starch granules had significantly different chemical compositions and gelatinization properties.
- Two starch granule-bound proteins (SGP), SGP-140 and SGP-145 were preferentially associated with A-type starch granules in developing and mature wheat endosperm.

- The preferential association of SGP-140 and SGP-145 with A-type starch granules was correlated to the size growth of wheat A-type starch granules, and related to the occurrence of the bimodal starch granule size-distribution in wheat, barley, rye and tritcale endosperm.
- SGP-140 and SGP-145 were different variants of SBEIc (a 152 kD isoform of wheat starch branching enzymes), and localized in the endosperm starch granules.
- Two nearly full-length cDNA, *wSsl-I* and *wSsl-II*, were cloned from a wheat endosperm cDNA library. Expressed in *E. coli*, *wSsl* cDNA produced an active starch synthase.
- wSSI was present at a constant level in starch granules through endosperm development, while the concentration of wSSI in endosperm soluble fractions varied with the growth of endosperm.
- Analysis based on single wheat kernel showed wSSI has similar concentrations in endosperm soluble fractions from 15 to 25 DPA, and much more wSSI was present in starch granules than in endosperm soluble fractions.
- wSSI contributed about two thirds of total soluble starch synthase activity in wheat endosperm.
- *wSsl* transcript was detected in leaf, stem, root, pre-anthesis floret, ovary and pollen. In endosperm, the expression level of *wSsl* at 5 – 10 DPA was higher than at 15 – 25 DPA.

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